# The orphan 7TM protein GPR50 as a novel regulator of TGF$\beta$ signal transduction

Stéfanie Wojciech

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**Stefanie WOJCIECH**

# The orphan 7TM protein GPR50 as a novel regulator of transforming growth factor β signal transduction

Directeur de thèse : Dr Ralf JOCKERS, Directeur de Recherche (Institut Cochin, Paris)

**JURY**

| | |
|---|---|
| Pr Mohammed TAOUIS | Président |
| Pr Olivier HERMINE | Rapporteur |
| Dr Laurent PRÉZEAU | Rapporteur |
| Dr Céline PRUNIER | Examinateur |
| Dr Philippe DELAGRANGE | Examinateur |
| Dr Mark SCOTT | Examinateur |

**Résumé**

**La protéine à 7TM GPR50 : un nouveau régulateur de la voie de signalisation TGFβ**

La protéine GPR50, qui fait partie de la famille des récepteurs de la mélatonine, est classée, avec une centaine d'autres protéines à sept domaines transmembranaires (7TM), dans la catégorie des récepteurs couplés aux protéines G hétérotrimériques (RCPG) orphelins, c'est-à-dire pour lesquels aucun ligand n'a pu être identifié. De plus en plus d'études montrent que les 7TM peuvent avoir des fonctions indépendantes d'un ligand. C'est le cas de GPR50 qui inhibe les fonctions du récepteur de la mélatonine $MT_1$ en interagissant directement avec lui. Nous avons cherché à identifier d'autres partenaires associés à GPR50 en appliquant la technique de purification par affinité en tandem et avons mis en évidence son interaction avec un récepteur du facteur de croissance *Transforming Growth Factor ß* (TGFβ), le récepteur de type I (TβRI).

Nous décrivons ici la formation d'un complexe entre GPR50 et le récepteur TβRI au niveau de la membrane plasmique, avec pour conséquence l'induction d'une activité constitutive du récepteur et des voies de signalisation en aval en l'absence de TGFβ, mais également en l'absence du récepteur TßRII qui est habituellement indispensable pour l'activation de TβRI par phosphorylation. Cette activité constitutive se traduit par la phosphorylation des protéines Smad2 et Smad3, leur intégration dans un complexe avec Smad4, la translocation du complexe dans le noyau et finalement l'activation de la transcription de leurs gènes-cibles. Nous avons décrypté les mécanismes moléculaires de cette activation constitutive en montrant que GPR50 entre en compétition, pour l'interaction avec TβRI, avec le régulateur négatif FKBP12, une protéine inhibitrice de l'activité basale du récepteur en l'absence de ligand. Nous avons identifié dans la queue intracytoplasmique de GPR50 un motif répétitif similaire à la séquence de FKBP12 impliquée dans son interaction avec TβRI, motif qui constitue la base moléculaire de cette compétition.

Nous avons étudié les conséquences fonctionnelles de cette activation en surexprimant GPR50 de manière stable dans la lignée cellulaire MDA-MB-231, dérivée d'un cancer de sein. Nous avons observé dans ces cellules des effets pro-migratoires et anti-prolifératifs similaires à ceux causés par l'administration de TGFβ.

En conclusion, ce travail décrit un nouveau mode d'activation du récepteur TβRI en l'absence de ligand, mais identifie également une nouvelle fonction indépendante d'un ligand pour le RCPG orphelin GPR50. En perspective de ce travail, nous allons essayer d'identifier des conditions biologiques où cette interaction pourrait prendre place afin de confirmer ces résultats dans un contexte plus physiologique.

Mots clés : RCPG orphelin, GPR50, voie de signalisation TGFβ, TβRI

**Abstract**

**The orphan 7TM protein GPR50 as a novel regulator of TGFβ signalling**

During the last years, it became more and more accepted that orphan G Protein coupled receptors (GPCRs) with a transmembrane spanning heptahelical core (7TM) can have ligand-independent functions. One of those 100 orphan GPCRs is GPR50, a 7TM protein with a long cytosolic domain. Recently, studies revealed ligand-independent functions for GPR50, where it has the capacity to modulate the activity of other proteins upon complex formation. By applying a tandem affinity purification approach we sought to identify further putative interacting partners of GPR50. One of the identified binding partners is the transforming growth factor β (TGFβ) receptor type I (TβRI).

The TGFβ-dependent signal transduction pathway of serine/threonine kinases is a pathway with direct signal flow from ligand over the receptor to its substrates, the Smads which translocate into nucleus where they bind DNA and regulate gene expression. An important question concerns the generation of specificity and fine-tuning of TGFβ-dependent signaling. Throughout the years, an important number of proteins which regulate the activity of the TGFβ signal transduction pathway in a positive or negative manner have been identified. Most of them act in a cell-context-dependent manner, allowing the regulation of TGFβ signaling adapted to the particular circumstances.

We report here the complex formation of GPR50 and TβRI on the plasma membrane. The consequence of this interaction is the GPR50-mediated induction of a constitutive activation of the TβRI and its downstream signaling in a TGFβ ligand-independent manner. This has been monitored by Smad2/3 phosphorylation, Smad2/3-Smad4 complex formation and their subsequent translocation into the nucleus, where they activate Smad-dependent gene expression. In order to decipher the molecular mechanism that allows this activation, we showed that GPR50 competes with the negative regulator, that prevents leaky TGFβ signaling, the gatekeeping molecule FKBP12, for binding to the TβRI. We identified a motif in FKBP12 involved in the interaction with TβRI with similarities to a motif in GPR50, providing a molecular basis for the replacement of FKBP12 by GPR50 in the TβRI complex. We showed that GPR50 is capable of activating the TβRI even in the absence of the TβRII, which normally is required for activating the TβRI by phosphorylation. This reveals a previously unknown mode of activation of the TβRI in absence of the TGFβ ligand and TβRII. In order to identify the functional consequences of this crosstalk, we studied migration and growth of MDA-MB-231 breast cancer cells stably overexpressing GPR50. In these cells, TGFβ-like pro-migratory and anti-proliferative effects have been observed.

Future research will help to identify tissues and biological circumstances, where this crosstalk could take place for putting this novel mode of regulation of TGFβ signaling pathway into a context-dependent-manner. Additionally our work established another ligand-independent task for the orphan 7TM protein GPR50, consolidating its function as binding partner and activity modulator.

**Key words**: orphan GPCR, GPR50, TGFβ signaling, TβRI

*« Être parisien, ce n'est pas être né à Paris, c'est y renaître. »*

*Sacha Guitry*

A ma famille

A mes amis

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# CONTENT

# Public presentation of work associated with this thesis

## Publications:

Benleulmi-Chaachoua A, **Wojciech S**, Jockers R *Les récepteurs couplés aux protéines G sous les feux de la rampe.* Biologie Aujourd'hui, 207, n°3, 2013

**Wojciech S,** Belaid-Choucair Z, Ahmad R, Journé AS, Daulat AM, Guillaume JL, Saade A, Clément N, Courivaud T, Tadagaki K, Delagrange P, Prunier C, Jockers R *Ligand-independent activation of type I TGFß receptor (TßRI) by the orphan 7-transmembrane protein GPR50 in the absence of the TßRII* (submitted)

## Oral communications:

**Wojciech S** *The orphan receptor GPR50 and its interaction with the TGFβ receptor I,* Journée des doctorants de l'Institut Cochin, February 2012, Paris.

**Wojciech S**, Belaid-Choucair Z, Saade A, Journé AS, Ahmad R, Daulat AM, Ferrand N, Guillaume JL, Clément N, Delagrange P, Prunier C, Jockers R *Crosstalk between GPR50 and Transforming Growth Factor β receptor I dependent signal transduction and its possible implication in cancer development*
FASEB SRC "Melatonin Biology: Actions & Therapeutics", July 2013, Niagara Falls, United States
→ selected for oral presentation from abstract

## Poster presentations:

**Wojciech S,** Saade A, Daulat AM, Ferrand N, Delagrange P, Clement N, Prunier C, Jockers R *The orphan G Protein-Coupled Receptor GPR50 influences TGFβ signaling by interacting with the TGFβ Receptor 1*
(1) FASEB SRC "TGF-β superfamily: signaling in development and disease", August 2011, Lucca, Italy

(2) Journée de l'école doctorale, September 2011, Le Kremlin Bicêtre

**Wojciech S,** Saade A, Journé AS, Daulat AM, Ferrand N, Delagrange P, Guillaume JL, Clement N, Prunier C, Jockers R *Cross-talk between the 7-transmembrane protein GPR50 and Transforming Growth Factor β receptor signal transduction*
(1) 1st Annual meeting of the GDR 3545, October 2012, Paris, France
→ recompensed by one out five poster prizes for young researchers
(2) Journée de l'école doctorale, September 2013, Le Kremlin Bicêtre

**Wojciech S**, Belaid-Choucair Z, Saade A, Journé AS, Ahmad R, Daulat AM, Ferrand N, Guillaume JL, Clement N, Delagrange P, Prunier C, Jockers R *The orphan 7TM GPR50 constitutively activates Transforming Growth Factor β receptor I and is possibly implicated in cancer development*
2nd annual meeting of the GDR 3545, October 2013, Strasbourg

# ABBREVIATIONS

| | |
|---|---|
| 5-HT | serotonin |
| 7TM | seven transmembrane domain |
| A | alanine |
| $A_{2A}$ | adenosine receptor subtype 2A |
| aa | amino acid |
| AKAP | A-kinase anchor proteins |
| Ala | alanine |
| ALK | activin receptor-like kinase |
| AP2 | adaptor protein 2 |
| ARE | activin-response element |
| ARF | activin-response factor |
| Arg | arginine |
| Asn | asparagine |
| Asp | aspartic acid |
| ATF3 | cyclic AMP-dependent transcription factor |
| Bcl2 | B-cell lymphoma 2 |
| BG | betaglycan |
| bHLH | basic helix-loop-helix |
| BIM | B-cell lymphoma L11 |
| BMP | bone morphogenic protein |
| BRET | bioluminescence resonance energy transfer |
| bZIP | basic leucine zipper |
| C | cysteine |
| c/EBP | CCAAT/enhancer-binding-protein |
| $Ca^{2+}$ | calcium ions |
| cAMP | cyclic adenosine monophosphat |
| CCL | CC chemokine ligand |
| Cdc42 | cell division control protein 42 |
| CDK | cyclin-dependent kinase |
| cDNA | complementary DNA |
| CNS | central nervous system |
| COS | **C**V-1 (simian) in **O**rigin carrying the **S**V40 |
| CpG | cytosine-guanine |
| CREB | cAMP response element-binding protein |
| CSF | cerebrospinal fluid |
| C-terminal | carboxy-terminal |
| CTGF | connective tissue growth factor |
| CXCR | CXC chemokine receptors |
| Cys | cysteine |
| D | aspartate |
| $D_2$ | dopamine receptor subtype 2 |
| DAF | TGFβ receptor homolog in *C.elegans* |
| DAG | diacylglycerol |
| DAPK | death-associated protein kinase |

| | |
|---|---|
| DAT | dopamine active transporter |
| Dlx | Homeoboxprotein Dlx |
| DNA | deoxyribonucleic acid |
| Dnmt | DNA-methyltransferase |
| DP1 | DP1 transcription factor |
| Dpr2 | dapper2 |
| DRAK2 | DAP kinase-related apoptosis-inducing protein kinase |
| E | glutamate |
| E2F4/5 | transcription factor |
| EBI2 | Epstein-Barr virus-induced G protein-coupled receptor 2 |
| EC domains | extracellular domains |
| ECL | extracellular loop |
| ECM | extracellular matrix |
| EEA | early endosome antigen |
| EGF | epidermal growth factor |
| ER | endoplasmatic reticulum |
| ETV | ETS family of transcription factors |
| ETV6-NTRK3 | fusion protein ETV6-NTRK3 |
| F | phenylalanine |
| FKBP12 | FK506-binding protein |
| FOP | *fibrodysplasia ossificans progressiva* |
| FoxH1 | FoxH transcription factor |
| FRAP | fluorescence recovery after photobleaching |
| FRET | Förster resonance energy transfer |
| FSH | follicle-stimulating hormone |
| G | glycine |
| GABA | gamma-aminobutyric acid |
| GADD34 | growth arrest- and DNA damage-inducible protein |
| GAP | GTPase-activating protein |
| GATA | GATA transcription factor |
| GDF | growth differentiation factor |
| GDP | guanosine diphosphate |
| GEF | guanine-nucleotide exchange factor |
| GIP | G-protein-coupled receptor interacting protein |
| Gln | glutamine |
| Glu | glutamate |
| Gly | glycine |
| GPCR | G protein-coupled receptor |
| GPS domain | GPCR proteolytic site |
| GR | glucocorticoid receptor |
| GRK | G protein-coupled receptor kinase |
| GS domain | gylcine/serine-rich domain |
| GSK | glycogen synthase kinase |
| GTP | guanosine triphosphate |
| GTPase | GTP hydrolyzing enzyme |
| H | histidine |

| | |
|---|---|
| HAT | histone acetylase |
| HDAC | histone deacetylase |
| HGF | hepatocyte growth factor |
| HIF | hypoxia inducible factor-1 |
| His | histidine |
| Hlx | homeoboxprotein Hlx |
| HNPCC | hereditary nonpolyposis colorectal cancer |
| HSC | hematopoietic stem cells |
| HTRF | homogeneous time resolved fluorescence |
| I | isoleucine |
| ICL | intracellular loops |
| ID | inhibitor of DNA binding |
| IGF | insulin-like growth factor |
| Ile | isoleucine |
| IP3 | inositol 1,4,5-triphosphate |
| JAK | Janus protein tyrosine kinase family |
| JNK | c-Jun N-terminal kinase |
| K | lysine |
| L | leucine |
| LAP | latency-associated peptide |
| LARG | Leukemia-associated RhoGEF |
| Leu | leucine |
| LH | luteinizing hormone |
| LPA | lysophosphatidic acid |
| LTBP | latent TGFβ binding protein |
| M | methionine |
| MAPK | mitogen-activated protein kinases |
| MH | Mad homology |
| miRNA | micro RNA |
| MIS | mullerian-inhibiting substance |
| MMTV | mouse mammary tumor virus |
| Mrg | Mas related gene |
| mRNA | messenger RNA |
| $MT_1$ | melatonin receptor subtype 1 |
| $MT_2$ | melatonin receptor subtype 2 |
| MUPP1 | multi-PDZ domain protein 1 |
| N | asparagine |
| NANOG | NANOG homeoboxprotein |
| NEDD4 | neural precursor cell expressed developmentally down-regulated protein 4 |
| NFκB | nuclear factor-kappa B |
| NGF | nerve growth factor |
| NHERF | $Na^+/H^+$ exchanger regulatory factor |
| NLS | nuclear localization signal/-sequence |
| Nogo-A | reticulon4/neurite outgrowth inhibitor |
| N-terminal | amino-terminal |
| NTRK | transmembrane surface receptor for neurotrophin-3 |

| | |
|---|---|
| Oct4 | octamer binding transcription factor 4 |
| ORL1 | opioid-receptor-like 1 |
| P | proline |
| p300/CBP | transcriptional co-activator of p300 and CREB-binding protein |
| p53 | protein 53 |
| PAC1 | procaspase activating compound 1 |
| PAR1 | thrombin receptor subtype PAR1 |
| PCR | polymerase chain reaction |
| PDGF | platelet-derived growth factor |
| PH domain | pleckstrin homolgy |
| Phe | phenylalanine |
| PI3K | phosphatidylinositide-3 kinase |
| PIP2 | phosphatidylinositol 4,5-bisphosphate |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospho lipase C |
| PP | protein phospatase |
| Pro | proline |
| PTEN | phosphatase and tensin homolog |
| Q | glutamine |
| R | arginine |
| RAFT | FKBP12-rapamycin associated protein |
| RAMP | receptor activity-modifying proteins |
| RANKL | receptor activator of NF-κB ligand |
| RGD | Arg-Gly-Asp sequence |
| RGS4 | regulator of G protein signaling 4 |
| RING | **R**eally **I**nteresting **N**ew **G**ene |
| RLP | Ras-like protein |
| RNA | ribonucleicacid |
| ROC1 | RING finger protein 1 |
| ROCK | Rho-associated protein kinase |
| RSTK | receptor serine/threonine kinase |
| RTK | receptor tyrosine kinase |
| Runx3 | Runx3 transcription factor |
| S | serine |
| S1P | sphingosine-1-phosphate |
| SARA | Smad anchor for receptor activation |
| SCN | suprachiasmatic nucleus |
| Ser | serine |
| SHC-1 | Src homology 2 domain-containing-transforming *protein* C1 |
| SID | Smad-interacting domain |
| SIM | Smad-interacting motif |
| Smad | small mothers against decapentaplegic |
| Smurf | Smad ubiquitin regulatory factor |
| SOX2 | SOX2 transcription factor |
| SP1 | SP1 transcription factor |

| | |
|---|---|
| SRF | serum-reponse factor |
| STAT | signal transducer and activator of transcription |
| STRAP | serine/threonine kinase receptor associated protein |
| SUMO | small ubiquitin-related modifier |
| SXS | Ser-x-Ser |
| T | threonine |
| TAB | TGFβ-activated protein kinase 1 (TAK1)-binding protein |
| TACE/ADAM17 | Tumor necrosis factor-alpha converting enzyme/metallopeptidase domain 17 |
| TAK | TGFβ-activated protein kinase |
| TAS | taste receptors |
| TFE3 | TFE3 transcription factor |
| TGFβ | transforming growth factor beta |
| Thr | threonine |
| TIC | tumor-initiating-cell |
| TIP60 | Tat-interactive protein 60 |
| TNF-α | tumor necrosis factor alpha |
| TRAF6 | TNF receptor associated factor |
| TrkA/B/C | neurotrophin NT-3 receptors A B and C |
| TRP | tryptophan |
| TSH | thyroid-stimulating hormone |
| Tyr | tyrosine |
| TβRI | TGFβ receptor type I |
| TβRII | TGFβ receptor type II |
| TβRIII | TGFβ receptor type III |
| Ubc9 | SUMO-conjugating enzyme |
| UCH37 | ubiquitin carboxyl-terminal hydrolase 37 |
| UDP glucose | uridine diphosphate glucose |
| UL | unique long |
| USP15 | ubiquitin specific peptidase 15 |
| V | valine |
| Val | valine |
| VEGF | vascular endothelial growth factor |
| W | tryptophan |
| WD40 | try-asp-rich domain |
| WWP | Try-Try-Pro proteins |
| Y | tyrosine |
| YAP65 | Yes-associated protein |

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# PROLOGUE

Every organism from prokaryotes to high eukaryotes has the capacity to sense and to adapt to the environmental circumstances. This is the result of a multitude of molecular events that are accomplished by the means of cellular communication. Stimuli from outside the cell that range from light, ions and small organic substances over to more complex molecules as peptides and proteins are translated into an intracellular response via transmembrane proteins with a signal-integrating function. This process is termed signal transduction. Advances in research during the last years made it possible to identify the core signaling pathways that are the basis of cellular communication and biological function. These transmembrane proteins, which react upon perception of extracellular signals are grouped into main classes: (I) G protein-coupled receptors (GPCRs), which activate intracellular G proteins that pass the signal to their effectors; (II) receptors with an intrinsic tyrosine kinase (RTKs) or serine/threonine kinase activity (RSTKs) which modulate the activity of intracellular proteins through their covalent modification by phosphorylation; (III) ion channels which modulate the cellular charge through ion in- or outflow; (IV) an exception are nuclear receptors, which are localized in the cytoplasm where they capture the signal from lipophilic molecules that pass through the plasma membrane into the cell. The conversion into an intracellular signal can be accomplished through various ways, like activation of second messenger molecules (cyclic adenosine monophosphate cAMP, calcium ions $Ca^{2+}$, nitrogen monoxide NO), induction of activation cascades (like the RTK – Ras – mitogen-activated-protein MAP kinase cascade) or the change of intracellular charge and pH. They all often converge in transforming the response on the nuclear level into the transcription of genes and their products respond to the extracellular stimulus and allow adaption to the environmental circumstances.

After the core signal transduction pathways and their components had been elucidated during the last century, the focus changed on the identification of pathway-associated proteins which modulate principal signaling. These proteins are necessary to allow the core pathway to adapt a context-specific signaling that fits the requirements of each cell type and the current biological conditions. In the age of “omics”, emerging high throughput screening methods facilitated the uncovering of pathway associated- and interacting molecules. Identification of their function often coincides with discovery of novel regulatory mechanisms, which form the basis of common principles in biology, and the revelation of intersection points between different signal transduction pathways. It becomes increasingly appreciated that, instead of

functioning independently apart, signal transduction pathways are part of a cellular signaling network. Thus, a cell responds in a certain cellular context or under specific physiological conditions by an adaptation of its signaling upon choosing the appropriate tools out of its repertoire.

The identification of further unexpected protein functions, interplays between the different signal transduction pathways and the elucidation of common principles of biology will help to gain more precise molecular explanations for the functioning of biological systems in health and disease. This knowledge can contribute to the identification of novel drug targets and the development of alternative strategies for disease treatment.

# I. INTRODUCTION

## 1. G protein-coupled receptors (GPCRs)

The family of G protein-coupled receptors (GPCRs) forms the largest entity of receptors mediating signal transduction from outside the cell through the plasma membrane into the cytosol. In humans they are encoded by about 800 genes, accounting for 3 to 4% of the entire genome (Vassilatis et al, 2003).

GPCRs convey the cellular response for an immense variety of ligands, including hormones, neurotransmitters, lipid compounds, chemokines, ions, photons, small organic molecules and nucleotides and also tastes and odorants (Figure 1). This diversity of ligands reflects the important role of GPCRs to manage diverse physiological processes as vision, smell and taste and to regulate neurological, cardiovascular, immune, endocrine and reproductive functions in the organism (Bockaert & Pin, 1999). Consequently, GPCRs are a notable therapeutic target, which is underlined by the fact that about 30% of the pharmaceuticals on the market direct GPCRs (Overington et al, 2006; Tyndall & Sandilya, 2005)

In recent years the research on GPCRs has shown an impressing dynamic due to structural resolutions, which led to an increase in understanding the mechanisms of GPCR signaling and could shed light on the various functional capacities of GPCRs. In 2012, these achievements have been recompensed by the Nobel prize in chemistry for two of the pioneers in GPCR research, Brian Kobilka and Robert Lefkowitz (Benovic, 2012; Bockaert, 2012).

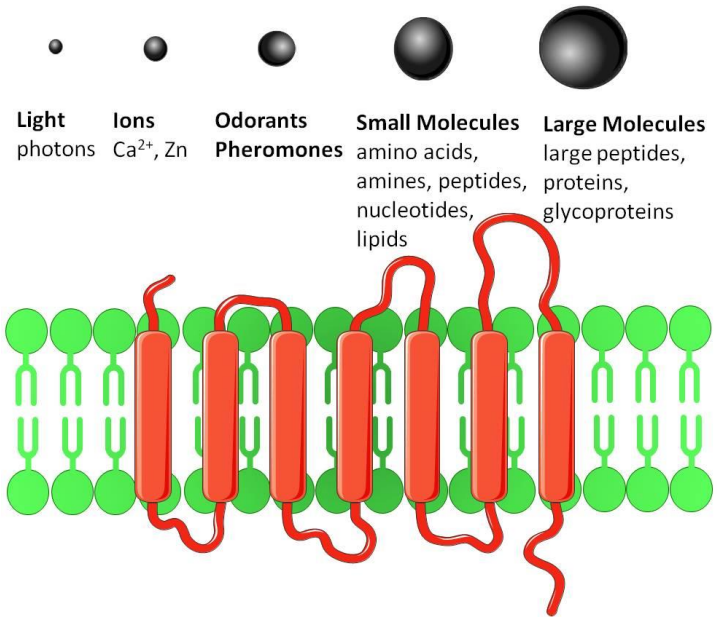


**Figure 1 Variety of GPCR ligands (Bockaert & Pin, 1999)**

GPCRs are transmembrane proteins that can bind a multitude of different ligands, including large molecules as proteins, different small molecules, as peptides, amino acids, amines, lipids, nucleotides and also percept signals in form of ions, photons from light or odorants and pheromones.

## 1.1 Structure of GPCRs

Despite the variety of ligands and GPCRs sizes that range from 300 to 1200 amino acids (Baldwin, 1993), all GPCRs share a common structural core. Rhodopsin sequencing in the 70's and cloning of other GPCRs in the late 80's (Dixon et al, 1986) could prove the existence of a shared topology for hormone receptors: all GPCRs are composed of seven transmembrane (7TM) spanning helical domains (see Figure 2 for details). The TM region is preceded by an N-terminus of variable length that can be involved in ligand binding and sometimes presents long sequences that form separate extracellular domains (EC domains) that also contribute to anchoring GPCRs to the extracellular matrix (ECM). TM segments are linked by three extracellular loops (ECLs) that play a role in ligand binding and three intracellular parts (ICLs) that are involved in downstream signaling. Furthermore, a cytosolic part in the C-terminus, including the helix #8 (the only known exception is CXCR4) is responsible establishing downstream signaling and functions as regulatory element in being a target for phosphorylation or a scaffold for binding other proteins. The sequence of the TM domain is quite conserved, while the other parts are more variable and form the basis of specificity concerning ligand binding, downstream signaling and the binding of modulators.

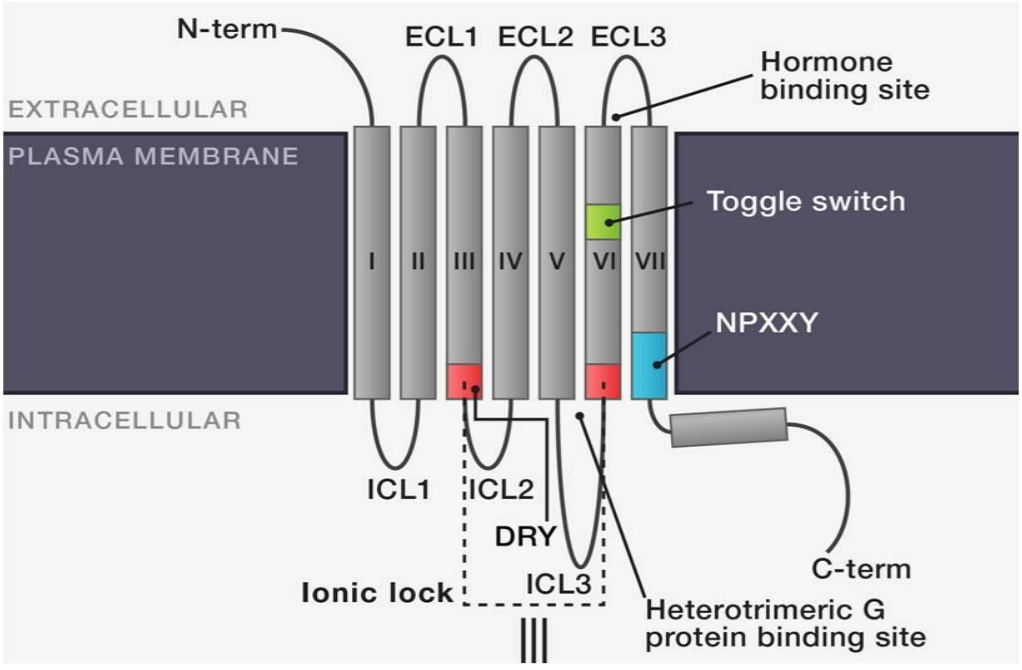


**Figure 2 GPCR topology with important structural features of class A GPCRs (Audet & Bouvier, 2012)**

The common topological core of all GPCRs are the seven transmembrane helices with the extracellular N-terminus and interhelical ECLs loops. ICLs loops between the helices range into the cytosol, where also the C-terminal part is located. Frequent features of class A GPCRs are the DRY motif in TM3 that forms an ionic lock with TM6, the toggle switch and the NPXXY sequence in the TM7. Ligand binding is often maintained by cooperation of ECL3 and TM6.

After deciphering the principal structure, further insight into GPCR structure was provided by the crystallization of bovine Rhodopsin, the prototypical GPCR, in 2000. (Palczewski et al, 2000). A breakthrough came in 2007 owing to the techniques that had been developed in the previous years that facilitate the obtainment of crystal structures (Cherezov et al, 2010). Application of this methods to the GPCR field resulted in discovery of the structure of the inactive adrenergic receptor $\beta_2$ in 2007 (Rasmussen et al, 2007; Rosenbaum et al, 2007), as shown in Figure 3, and $\beta_1$ in 2008 (Warne et al, 2008) bound either to an inverse agonist or an antagonist respectively. It was quickly followed by the structure of ligand-free rhodopsin (opsin) (Park et al, 2008; Scheerer et al, 2008). Further advances allowed the visualization of active structures as for the agonist-bound adenosine $A_{2A}$ receptor (Lebon et al, 2011; Xu et al, 2011) and the $\beta_2$ adrenergic receptor in complex with the heterotrimeric G proteins (Rasmussen et al, 2011b).

These and other structures in complex with ligand or coupled to G proteins or β-arrestins led the way from a simple observation of the structure to comparative analysis of the structural changes depending on the different conformations of GPCRs.

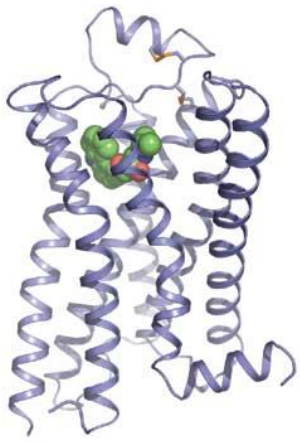


**Figure 3 Crystal structure of $\beta_2$ adrenergic receptor (Rasmussen et al, 2007)**

The crystal structure of the $\beta_2$ adrengic receptor bound to an inverse agonist for stabilization of the inactive conformation.

Currently, more than 20 class A GPCR structures have been obtained (Katritch et al, 2013). The structural identification of other GPCR families has progressed slower, but first results were achieved with the structure of Smoothened (Wang et al, 2013), extracellular parts of the glutamate receptor $mGluR_7$ (Muto et al, 2007) and class B family members (Hollenstein et al, 2013; Siu et al, 2013). This has helped to identify specific characteristics of each family and to describe the structural diversity in the different GPCR classes (Venkatakrishnan et al, 2013). The beginning of this structure resolution era in GPCRs helped to gain information about the flexibility and functionality of GPCRs and revealed structure-function relations.

## 1.2 Families of GPCRs

Aside from their common transmembrane core structure, GPCRs vary in their other structural elements. Analysis of shared features led to a first categorization into four different main classes as shown in Table 1 (Kolakowski, 1994).

**Table 1 Classification of GPCRs (Lagerstrom & Schioth, 2008)**

Current classification of GPCRs (after (Kolakowski, 1994)), with the number of proteins they comprise and the chemical nature of their ligands.

| | Class A | Class B | | Class C | Others | |
|---|---|---|---|---|---|---|
| **Property** | **Rhodopsin** | **Secretin** | **Adhesion** | **Glutamate** | **Frizzled** | **Taste2** |
| Number of full-length receptor proteins* | 672 (388 ORs) | 15 | 33 | 22 | 11 | 25 |
| Number of identified major drug targets‡ | >39 | 4 | 0 | 3 | 0 | 0 |
| Number of orphans* | 63 (not including ORs) | 0 | 30 | 7 | 0 | 21 |
| Type of ligand | Peptides, proteins (including enzymes), small organic compounds, lipid-like substances, nucleotides | Peptides, proteins | Proteins, glycosaminoglycan | Amino acids, cations, small organic compounds, carbohydrates | Proteins | Small organic compounds |

### Class A – rhodopsin like GPCRs

The class A is the largest GPCR family with approximately 700 members, which vary a lot in primary structure and ligand specificity. Their common feature is the short extracellular N-terminal part and conserved regions in the 7TM domain. In TM3 is a D/ERY motif, which is also part of the inactivating ionic lock with a Glu-residue in TM6, two cysteines that form a disulfide-bond between ECL2 and TM3 and a NPXXY motif that is located at the end of TM7

attenuating the helical structure and forming a water pocket, as illustrated in Figure 2 (Katritch et al, 2012). Further analysis enabled the formation of subgroups among class A GPCRs: (I) Subclass α regroups receptors that preferentially bind small ligands, which occurs in the 7TM region between TM3 and TM6. (II) Subclass β is responsible for the binding of large peptide ligands. (III) Subclass γ is composed of receptors for peptides and lipidic substances. (IV) Subclass δ GPCRs bind either nucleotides and glycoproteins or some of them are responsible for the perception of odorants and taste. The large amount of nearly 400 olfactory receptors found in humans is also part of this class. In addition to these 4 subclasses, another organization model exists, which divides class A into 19 subfamilies that have common characteristic features and often bind similar ligands, like the of melatonin receptors family (see Chapter 2).

**Class B – adhesion and secretin GPCRs**

This family comprises two different groups of GPCRs:

(1) Adhesion GPCRs

This family consists of about 30 members. They display unique elements, like their long N-termini that are often glycosylated. This N-terminal part is often composed of distinct domains, which are also found in other proteins with long extracellular parts like the cadherin-, lectin-, IgG- and EGF domains, which serve for binding ECM components and maintain ligand-receptor-interactions. Some receptors in this family bind proteins and glycosaminoglycans, but most are still orphan receptors (Gupte et al, 2012; Paavola & Hall, 2012).

(2) Secretin family of GPCRs

These 15 family members bind large ligands, such as peptides and proteins, with their extracellular domain. Since these ligands are important players in the maintenance of organism homeostasis, they form an attractive drug target.

**Class C – The glutamate family of metabotropic receptors**

The glutamate family of GPCRs is comprised of 25 members with amino acid ligands, such as glutamate and GABA, small organic compounds, and the cation $Ca^{2+}$. Among them are also three taste receptors. Their characteristic feature is their extended N-terminus that is implicated in ligand binding. Crystal structure analyses demonstrate a possible venus flytrap mechanism of this domain (Muto et al, 2007).

**Others – frizzled GPCRs and taste receptors (TAS)**

This class is composed of 11 frizzled receptors of which are 10 that bind the frizzled ligand, which is the glycoprotein Wnt, and one Smoothened ligand binding receptor.

In this class of receptors are also 25 taste receptors (TAS) that bind small organic compounds.

## 1.3 Signal transduction of GPCRs

The evidence that GPCRs couple to G proteins was recognized in 1970 as Martin Rodbell discovered that incubation of cells with glucagon in the presence of GTP triggers the activation of the adenylate cyclase. This led to a reformation of the concept of the signal flow of hormone receptors going from ligand via the receptor over to the G protein and its effector. In addition the name "G protein-coupled receptor" for this group of signal transducing membrane proteins became established.

### 1.3.1 Structural basis of GPCR activation

Ligand binding to a GPCR induces conformational changes which consequently affect its activation state. Experimental findings and structural analysis during the last years gave more insight into the complexity and the different facets of GPCR activation. This led to the shift in seeing GPCR activation as a process implicating a continuum of conformations instead of a simple two-state switch.

The resolution of GPCR structure has aided in revealing the conformational changes that occur upon activation. Surprisingly, it was found that there is not only one active conformation, but several different ones depending on the progress of activation (Figure 4).

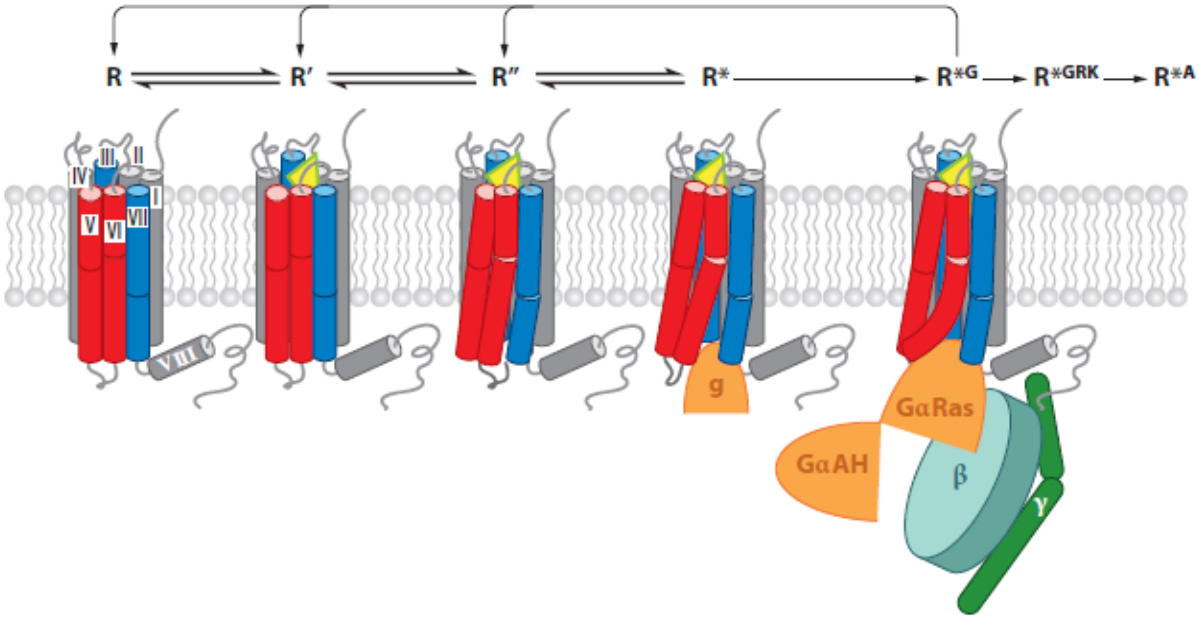



**Figure 4 Activation mechanism and conformational rearrangements of GPCRs (Katritch et al, 2013)**

R is the inactive ground state, small local changes occur in the inactive low-affinity agonist-bound state R'. Achieving R'' is accompanied by substantial conformational changes and rearrangements in the receptor, leading to at least partial exposition of the G protein binding site. R* is the activated state where the structural reorganization allows the interaction with the Gα protein. Finally, R*G is the fully active receptor conformation in complex with the heterotrimeric G protein. Additionally R*GRK and R*A are the conformations that receptors have upon the interaction with GRKs or β-arrestin. Noteworthy, most processes are bidirectional (indicated with flashes), while the formation of the R*G complex is unidirectional since it is accompanied by a non-reversible GTP hydrolysis. The TMs that are mainly implicated in the activation process are highlighted in red (5 and 6) and blue (3 and 7).

As a result of the bidirectionality of the different activation conformations shown in Figure 4, there is always equilibrium between receptors in the inactivated and in the activated states.

*Structural changes during the activation process*

During the activation process, the helices undergo several substantial rearrangements in the helical core in response to ligand binding. Ligand binding induces conformational changes that are propagated from the extracellular portion of the GPCR to the cytoplasmic surface. These conformational changes take place in several steps: ligand binding occurs upon interaction with ECLs and parts of the 7TM domain, the 7TM functions as stabilizing core, and the ICLs are responsible for the intracellular transformation of the response. For rhodopsin-like GPCRs, the movements during activation process have been shown for rhodopsin (Altenbach et al, 2008) and $\beta_2$ adrenergic receptors (Yao et al, 2006): in the inactive state, most of the class A members have an ionic lock between TM3 and TM6 that fix

the inactive conformation. Ligand binding then leads to a loss of the ionic lock and an important outward movement of TM5 and TM6, that creates a binding site for the Gα protein, allowing the active ternary complex formation and the transformation of ligand binding to an intracellular effect (Rosenbaum et al, 2009).

### 1.3.2 Downstream signaling of GPCRs

The initiating step in signal transduction of GPCRs is the binding of a ligand. Ligand binding is incredibly variable, occurs in various regions of the GPCR and each receptor has a binding site adapted to the structure of its ligand (reviewed in (Audet & Bouvier, 2012).

#### 1.3.2.1 Ligand efficacy

Beside this specificity in ligand binding, synthetic ligands of similar chemical structure to the natural ligand can differ in their strength to shift the receptor towards the state of full activation or even block its activation. The capacity of a ligand to activate a receptor is termed ligand efficacy. Every natural or synthetic ligand stabilizes an individual set of receptor conformations, which is reflected by its capacities to more or less fully activate or inhibit distinct downstream signaling pathways and biological responses. Different efficacy classes have been defined according to their effects on receptor activation (Figure 5):

(1) An agonist is a ligand that leads to the activation of GPCRs, either partial (= partial agonist) or completely (= full agonists).

(2) An antagonist is a ligand that inhibits receptor activity. Within this classification are inverse agonists, which inhibit spontaneous activity or counteract the basal activation of the receptor. Another type of antagonist are neutral antagonists, which are able to bind the receptor, but lack intrinsic activity, thus blocking the receptor binding site for agonists and inverse agonists (Rosenbaum et al, 2009).

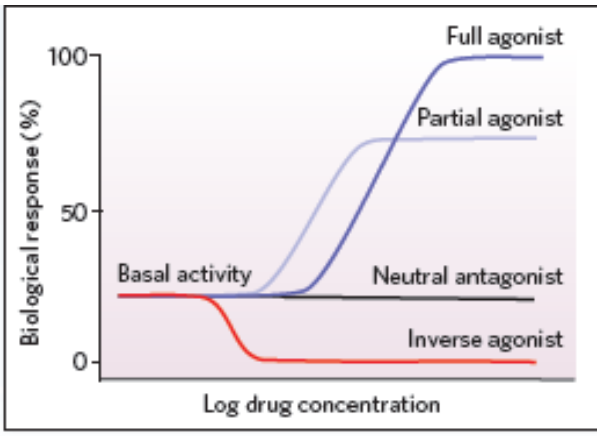



**Figure 5 Ligand efficacy and downstream signaling (Rosenbaum et al, 2009)**

It can be differentiated between agonists that activate receptors either partially (partial agonist, light blue) or fully (full agonist, dark blue). To counteract GPCR activity, there are neutral antagonists which block receptor access and biological response (black), while inverse agonists reduce basal activity (red).

*Constitutive activity of GPCRs*

In the case of constitutive receptor activity a significant proportion of the GPCR can shuttle towards active conformations even in the absence of ligand (Tiberi & Caron, 1994). It is described for several GPCRs and goes along with constant coupling to G proteins and downstream signaling. A structural explanation for class A receptors could be the absence of the inhibitory ionic lock, as in the melatonin receptors. Mutations in GPCRs, that shift the balance towards more activated than inactivated receptors are often associated with diseases (Seifert & Wenzel-Seifert, 2002).

#### 1.3.2.2 Signaling via G proteins

After ligand binding has induced conformational changes, the receptor is capable of transducing the signal to different intracellular effectors. Classical downstream signal transduction includes the heterotrimeric G proteins as mediators between receptor and effector. By binding to effectors, like enzymes or channels, they are responsible for intracellular signal transduction.

*Function and diversity of heterotrimeric G proteins*

The heterotrimeric G proteins belong to the family of guanine-nucleotide-binding proteins. They are highly conserved during evolution and composed of three subunits: α (about 40kDa), β (about 35kDa) and γ (about 8-10kDa), which can exist in different isoforms and

assemble in various combinations in the heterotrimeric G protein complex (Cabrera-Vera et al, 2003).

In the inactive state, the three G protein subunits are associated with the Gα subunit binding GDP. Following its recruitment by an activated GPCR, the Gα protein undergoes conformational changes leading to a GDP to GTP exchange. This activation step is accompanied by the dissociation of the Gβγ complex from Gα, what allows the subsequent interaction with their corresponding effectors. Termination of G protein signaling occurs through GTP hydrolysis, causing its dissociation from the effector and a reassembly of the three subunits, which are ready for another activation-inactivation cycle.

The capacity of each subunit to interact with different effectors through their protein interacting domains allows a broad spectrum of downstream signaling pathways (Kostenis et al, 2005). The Gα proteins can be divided into four subfamilies with specific downstream signal transduction (see Figure 6 for overview):

$G\alpha_s$ and $G\alpha_i$ proteins either stimulate ($G\alpha_s$) or inhibit ($G\alpha_i$) adenylate cyclaces that convert ATP into cyclic AMP (cAMP). cAMP functions as a second messenger, activates protein kinase A (PKA), which in turn phosphorylates and activates the transcription factor cAMP response element protein (CREB), which regulates the expression of numerous genes.

$G\alpha_q$ proteins activate the phospho lipase C (PLC) that hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to diacylglycerol (DAG) and inositoltriphosphate ($IP_3$). DAG can activate the protein kinase C (PKC) and $IP_3$ binds to receptors which triggers the release of calcium from internal stores.

$G\alpha_{12/13}$ proteins can direct downstream signaling towards the regulation of Rho GTPase activity.

The Gβ and γ subunits remain associated after dissociation from the Gα protein, and interact and activate $PLC_\beta$ through their pleckstrin-homology (PH) domains, which triggers IP3 and DAG production, like the $G\alpha_q$ proteins. Additionally they can also activate ion channels and phosphatidylinositide 3 (PI3) -kinases.

Other possible actions of G protein subunits have been reported like guanylyl cyclase activation for cGMP generation, regulation of mitogen-activated protein (MAP) kinase activity and the activation of phosphatases, highlighting the diversity of G protein targets inside the cell (Milligan & Kostenis, 2006).

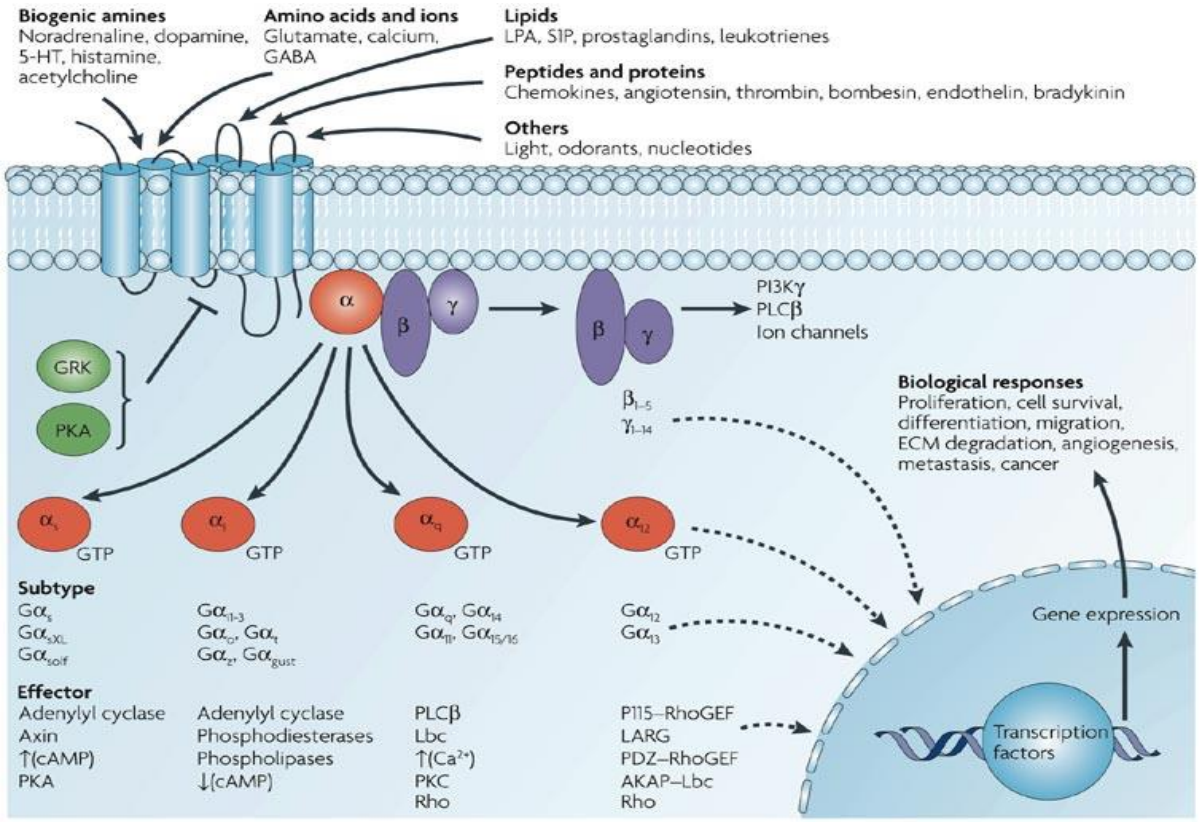



**Figure 6 Signaling of GPCRs via heterotrimeric G proteins (Dorsam & Gutkind, 2007)**

The heteromeric G proteins can direct GPCR signals depending on their subtype towards different effectors. The different Gα proteins are indicated in red and the βγ subunit in purple.

*Structural basis of G protein coupling to the receptor*

The activation of G proteins requires their coupling to the receptor, which is also subject to conformational rearrangements in the receptor. Ligand binding induces the loss of the ionic lock between TM3 and TM6 and the outward movement of TM6 creates a binding site for the G protein. Thus, a conformation, that allows the binding of the G protein is stabilized (Audet & Bouvier, 2012). More specific insights for the receptor-G protein interaction were recently gained with $\beta_2$ adrenergic crystal structures bound to the G protein (Rasmussen et al, 2011a; Rasmussen et al, 2011b): Gα protein interaction sites are found on TM3, TM5, TM6, and the ICL2 of the receptor. After coupling of G proteins to an active receptor, the nucleotide exchange from GDP to GTP for activation is facilitated through structural adjustments and interactions between the receptor and the G protein (Bouvier, 2013).

But the different existing G protein isoforms and the specificity of each ligand and its ability to signal via different Gα protein suggest the existence of additional mechanisms to achieve the pairing of a ligand with its specific G protein. It will be a future challenge to describe the mechanisms that allow the recognition of ligand and associated G protein via the 7TM core.

*Regulation of G protein activity*

Different classes of proteins control G protein activity in order to regulate GPCR-dependent signal transduction. Some proteins can act on the GDP-to-GTP exchange: while guanine-nucleotide-exchange factors (GEFs) are activating, GDP-dissociation inhibitors (GDIs) have an inhibiting function. Activators-of-G protein-signaling (AGS) proteins bind to the βγ subunit, to form a quaternary complex (Cismowski, 2006). RGS proteins are regulators of G protein activity that mediate their inactivation by promoting GTP hydrolysis, thus functioning like a GTPase-activating protein (GAP). Their expression is often induced via a negative feedback loop mechanism (Ross & Wilkie, 2000).

#### 1.3.2.3 G protein-independent signaling

Different G protein-independent pathways have been discovered in the recent years: GPCRs can function via the β-arrestin coupling that favors the activation of MAP kinases like extracellular-regulated-kinase ERK (see below). GPCRs can also bind to PDZ domain-containing proteins like MUPP1 that modulate their activity and localization (Guillaume et al, 2008). The existence of residues that are phosphorylatable can form a binding motif for SH2 domain proteins like Src and trigger associated downstream signaling events (Marinissen & Gutkind, 2001). Upcoming research will surely uncover more presently unexpected downstream signaling activities in the absence of G proteins.

### 1.3.3 Regulation of GPCR activity

Beside above described proteins that act on the G proteins, further distinct mechanisms to modulate GPCR activity and signaling:

#### 1.3.3.1 Receptor trafficking and desensitization

The receptor desensitization claims to stop ligand binding or to prevent the receptor from inducing downstream signaling in response to ligand binding. There exists a canonical way to stimulate receptor desensitization.

*Receptor phosphorylation*

The first event to stop ligand-dependent effects is a phosphorylation on serine/threonine residues in the cytosolic C-terminus of the receptor. Different kinases have been identified to

target GPCRs, like PKA, PKC and GPCR kinases, the GRKs. Seven different GRKs have been identified and all are activated as a downstream signaling event or by the binding of GPCR effectors or second messengers. GRKs also have a GAP domain to inactivate G proteins (Luttrell & Lefkowitz, 2002). Typically, GRK-mediated phosphorylation leads to a decoupling of G proteins, but can also trigger other signaling cascades (Zanello et al, 1997).

*β-arrestin recruitment*

Phosphorylation is a prerequisite not sufficient for inactivation of GPCRs, but it creates a binding site for β-arrestin. β-arrestins are responsible for receptor internalization by interacting with the adaptor protein AP2 in the endosomes and triggering the formation of receptor-containing vesicles that translocate inside the cell. Afterwards, receptors are either recycled and inactive receptors shuttle back to the plasma membrane, or otherwise, they can also be directed to the lysosomes to be proteolyzed (Luttrell & Lefkowitz, 2002).

*β-arrestin-mediated G protein-independent signaling*

Besides blocking further G protein docking and activation of GPCRs, β-arrestin recruitment can also have other possible outcomes. They can function as scaffolding proteins for diesterases that degrade cAMP and terminate signaling, ubiquitin ligases for a proteasomal degradation of the receptor complex (Shenoy & Lefkowitz, 2011), or trigger the activation of other pathways by activation of Src (Luttrell et al, 1999) or the PI3K/Akt pathway.

The β-arrestin-mediated internalization into endosomes can also help for the creation of signaling endosomes and prompt ERK activation (DeFea et al, 2000).

In addition to β-arrestin actions on GPCRs, increasing evidence suggests their involvement in other GPCR-independent pathways, as the TGFβ signaling pathway (Chen et al, 2003; McLean et al, 2013).

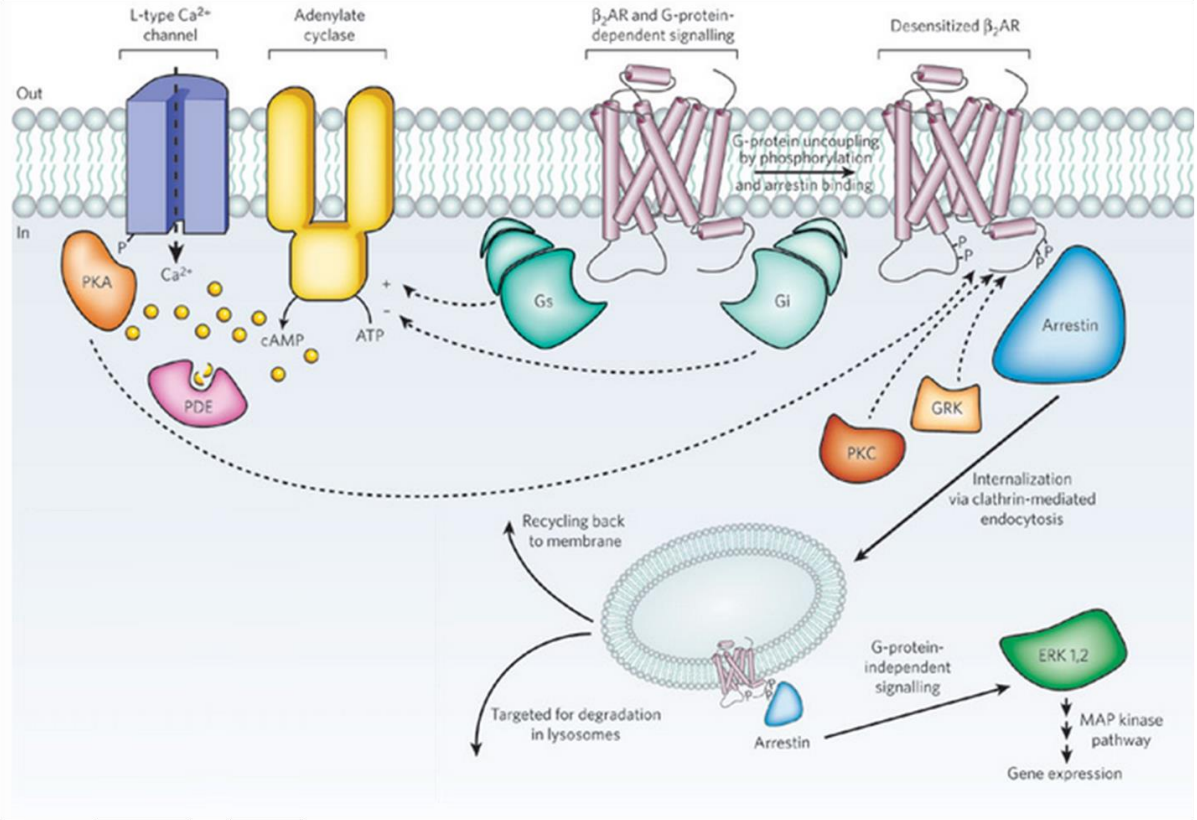



**Figure 7 Signal transduction of the $\beta_2$ adrenergic receptor (Rosenbaum et al, 2009)**

The $\beta_2$ adrenergic receptor can mediate different downstream signaling pathways. Dependent on the bound ligand, it can activate $G\alpha_i$ or $G\alpha_s$ proteins that either activate or inhibit cAMP production. The subsequent phosphorylation of PKA triggers the activation of $Ca^{2+}$ channels. The recruitment of β-arrestin to the receptor can induce different scenarios: a bound phosphodiesterase can attenuate adenylate cyclase activity, the MAP Kinases ERK1 and ERK2 can be activated or the receptor can be internalized with subsequent recycling or lysosomal degradation.

#### 1.3.3.2 Regulation of GPCR signaling by interacting proteins

The activity of GPCRs can also be regulated by different classes of interacting proteins:

GPCR-interacting proteins (GIPS) comprise GRKs and β-arrestin, but a lot of other molecules have been identified that can participate in regulation of GPCR localization and activity after complex formation (Magalhaes et al, 2012).

A specific class of proteins are receptor-activity modifying proteins (RAMPs), which were found to interact with the calcitonin receptor and alter its activity and pharmacology (Hay et al, 2006). Other proteins are GPCR-associated sorting proteins (GASPs) that play a role in subcellular localization or influence the trafficking towards the plasma membrane after synthesis.

The PDZ motif in the C-terminal part of the GPCR can mediate the interaction with PDZ proteins like NHERF or MUPP1 that can be required for GPCR activation (Ritter & Hall, 2009).
Additionally, a lot of other proteins exist to regulate activity, pharmacology and localization of GPCRs and are described in more detail in the literature (Maurice et al, 2011a).

### 1.3.4 Diversity and special features of GPCR signaling

The preceding chapter illustrates that one GPCR can adopt different conformations. In dependence of each natural or synthetic ligand, a distinct conformation with distinct downstream signaling activity is obtained. This diversity of GPCR signaling can be further increased by the following opportunities of GPCR signal transduction:

#### 1.3.4.1 Allosteric regulation of GPCR activity

Allosterism was firstly described for enzymes in 1963 (Monod et al, 1963). This means a compound can bind to an allosteric site in the receptor, which is another than the standard, the orthosteric one. Thus, an allosteric ligand can influence receptor actions or have an effect on the orthosteric ligand activity (Bridges & Lindsley, 2008). The binding of an allosteric molecule leads to conformational changes, compared to the orthosteric-ligand-bound-only-state and is often accompanied by a different signaling outcome. Hence, allosteric ligands also play a role in biased signaling (see below).
Allosteric ligands can be different types of molecules: small compounds but also proteins like interacting and regulating proteins described in the preceding chapter and they function either as positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs). The identification of allosteric modulators and their corresponding binding sites is one of the current major interests in GPCR research (Katritch et al, 2012). They bear a huge therapeutic potential for the development of a new type of synthetic drug for GPCRs and benefit from *in silico* drug design. Furthermore, bitopic ligands, that occupy more than one binding site in a receptor and thus display particular signaling profiles, are another future application of the allosterism concept to therapeutics (Valant et al, 2012). An example for a drug with an allosteric mode of action are the benzodiazepines binding to GABA receptors (Sebag & Pantel, 2012).

#### 1.3.4.2 Biased Signaling of GPCRs

The phenomenon of biased signaling is grounded in the ability of one GPCR to induce several downstream signaling pathways. Biased signaling describes the ability of one synthetic ligand to direct signaling into a particular pathway, compared to the natural ligand (Rajagopal et al, 2010).

The structural basis is the existence of several active conformations of one receptor (see 1.3.1.). This is also reflected in the ability of each ligand for one GPCR to stabilize a particular set of conformations implicating a specific rearrangement of certain parts in the receptor in response to ligand, which finds its consequence in a specific ligand-corresponding downstream signaling (Wacker et al, 2013). This capacity of a ligand to preferentially activate only one signal transduction pathway is termed "functional selectivity" (Rosenbaum 2009). Technical developments, like bioluminescence resonance energy transfer (BRET), permit to study conformational changes that underlie biased signaling (Galandrin et al, 2008).

Drug design takes advantage of biased signaling to find suitable ligands with impaired efficacy and reduced side effects (Kenakin & Christopoulos, 2013).

In addition to ligands, receptors can be biased, which is the case in mutated receptors as demonstrated for different variants of the melatonin receptor $MT_2$ (Bonnefond et al, 2012).

The existence of biased signaling supports the concept of multi-functionality of GPCRs and its action as a center of integration for different ligands by permitting each of them a specific signaling pathway and a different biological response.

#### 1.3.4.3 GPCR variants

With the development of high throughput sequencing to analyze genomic DNA and their application to genetic association studies, it was revealed, that most GPCRs exist in sequence variants or display frequent germline mutations (SNPs, single nucleotide polymorphisms) (Nelson et al, 2012). These variants are sometimes found associated with a higher risk to develop certain diseases. One pioneer study tried to find a connection of variants in melatonin receptor genes and the risk to develop type 2 diabetes, these variants are either located in intronic sequences (Andersson et al, 2010; Bouatia-Naji et al, 2009; Chambers et al, 2009; Sparso et al, 2009) or the coding exon region of the $MT_2$ receptor gene MTNR1B (Bonnefond et al, 2012). Variants in the coding region might introduce changes in the amino acid sequence that affect the functionality of the protein. The interesting task is now, to

characterize the signaling properties of each of those variants and to identify their potential for biased signaling.

Altogether, these findings gave rise to the supposition that the SNPs we naturally find in everyone of us, might also evoke different signaling properties and functional effects of GPCRs and that this concept could be translated to other receptors and other proteins. In turn, this can explain why certain people have a higher tendency to develop certain disorders and different sensitivities to medication. To generalize, this concept might not only find its application in disease but could also serve as the scientific basis to understand the individuality of everyone.

## 1.4 GPCR Homo- and heteromerisation

GPCR homo- and heteromerisation means the assembly of two or more monomers into one functional complex, either with the same (di- or oligo-homomer) or with different monomers (di- or oligo- heteromer) of other GPCRs.

### 1.4.1 Homodi/oligo-merisation of GPCRs

The existence of receptor dimers and oligomers is a quite young concept in GPCR research, while for several other receptor classes dimerisation is indispensable for signaling activity. It is described for different receptor tyrosine kinases (RTKs) like the epidermal-growth-factor receptor (EGFR) (Blakely et al, 2000), serine/threonine receptor kinases as the transforming growth factor β (TGFβ) receptor (Franzen et al, 1993), cytokine receptors that are coupled to soluble tyrosine kinases as found in the januskinase (JAK) - signal transducers and activators of transcription (STAT) pathway or even nuclear receptors (Marianayagam et al, 2004). The reason is that their ligands are dimeric and have two symmetric binding surfaces that require binding to two receptors. This often goes along with the fact, that receptor activation requires transphosphorylation from one receptor monomer to the other. For GPCRs, the existence of higher-order structures slowly became accepted with turning of the last century (Bouvier, 2001; Hebert & Bouvier, 1998; Salahpour et al, 2000). A list of GPCRs for whom the existence of multimeric states has been proven can be found at http://data.gpcr-okb.org/gpcr-okb/oligomer/list

#### 1.4.1.1 GPCR homodimers

One receptor monomer is sufficient to perform signaling (Whorton et al, 2007) thus questioning the necessity of dimeric complexes. The development of appropriate biophysical techniques based on energy transfer between donor and acceptor molecules like BRET (Angers et al, 2000; Mercier et al, 2002) and homogeneous time resolved fluorescence (HTRF) (Maurel et al, 2004) have become a versatile tool to study and prove GPCR dimerisation. Further supporting evidence is provided by recent crystal structures, that propose the existence of dimer-interaction surfaces as shown for the opioid receptors (Granier, 2012). Consequently, the concept of receptor dimerisation is now established and it has been shown that it even can be mandatory for correct functioning, for example for class C GABA receptors (Galvez et al, 2001).

Evidence exists that an organization in dimers can be constitutive and that their formation already occurs during protein synthesis (Terrillon et al, 2003). The mechanisms of dimer formation are various and can rise from the formation of disulfide bridges between receptors over transmembrane interactions to coiled-coil interactions of helices in the cytosol (Bouvier, 2001).

#### 1.4.1.2 GPCR homooligomers

The existence of homo-oligomeric assembly of GPCRs has been shown by different techniques. Additionally to BRET and FRET, other light based studies like total internal reflection fluorescence microscope (TIRF) (Boyer & Slesinger, 2010), fluorescence recovery after photobleaching (FRAP) (Dorsch et al, 2009) or single molecule techniques (Calebiro et al, 2013) proposed the existence of higher-order states for GPCRs. The technique of HTRF also allowed their detection in native tissue (Albizu et al, 2010). These techniques brought evidence for GABA receptors being organized as tetramer (Maurel et al, 2008) and $M_3$ muscarinic receptors as hexamers (Patowary et al, 2013). The crystal structure of the turkey $\beta_1$ adrenergic receptors in a lipid membrane displayed oligomeric structures (Huang et al, 2013).

The functional effect of oligomerisation remains speculative. It might be a mean to facilitate ligand binding and signaling complex formation (Maurice et al, 2011b), but also could be a way for the receptor to stabilize inactive conformations (Bouvier, 2013).

### 1.4.2 Heteromerisation of GPCRs

GPCRs form high order structures by the assembly of different GPCR mono- or dimers, what is called heteromerisation.

*Examples of heteromer formation*

As for homomerisation, heteromers are assembled in the endoplasmatic reticulum (ER) before their insertion in the plasma membrane (Milligan, 2006). It can take place between different types of GPCRs:

(1) Heteromerisation in the same subfamily is often found between closely related GPCRs. The class C GABA receptor $GABA_{B1}$ and $GABA_{B2}$ form obligate heterodimers (Bettler et al, 2004; Kaupmann et al, 1998; White et al, 1998) based on their asymmetric mode of activation, where one monomer is responsible for ligand binding and the other manages the activation of the G protein (Pin et al, 2009). The example of opioid receptors shows a case where both monomers κ and δ are necessary for activation and a strong ligand binding (Jordan & Devi, 1999). In the taste receptor subfamily, $T1R_1$ and $T1R_2$ form heteromers with the $T1R_3$ ligands and their combination defines the taste we experience (Xu et al, 2004; Zhao et al, 2003).

(2) Heteromerisation in different, but related subfamilies of one class often involves receptor transactivation, where ligand binding to one receptor activates the other, despite their different ligand preferences. The heteromeric complex formation between the adenosine receptor $A_2$ and the dopamine receptor $D_2$ is an example of allosteric regulation of one receptor by the other (Ferre et al, 1991; Hillion et al, 2002). In contrast, heteromerisation can also have antagonistic effects, as in the case of glutamate binding to the complex of receptor $mGluR_2$ glutamate receptor and the $5\text{-}HT_{2A}$ serotonin receptor heteromer (Gonzalez-Maeso et al, 2008).

(3) Heteromerisation in different GPCR classes of families occurs less frequently, but has been shown for the adenosine $A_1$/glutamate $mGluR_{1\alpha}$ dimer composed of one class C and class A receptor (Ciruela et al, 2001).

Rare is the case where more than two different receptors assemble into oligomers, but the example of the heteromer of adenosine $A_{2A}$ and dopamine $D_2$ receptors with the glutamate receptor $mGluR_5$ (Cabello et al, 2009) proposes the existence of more complex heteromeric structures for GPCRs.

(4) The case of heterodimerisation of with orphan GPCRs will be discussed in more detail separately (see 1.6.2.)

*Heteromer specific signal transduction and effects*

Heteromer formation increases the spectrum of biological responses and signaling variety of GPCRs. It affects signaling either in an antagonistic, synergistic or receptor-biased fashion and can induce crossactivation or –inhibition (Smith & Milligan, 2010). Heterodimerisation can, for example, change the ligand binding properties. This is accomplished by either obligatory heteromers ($GABA_B$) or involves conditional allosteric regulation mechanisms where ligand binding to one receptor affects the affinity and efficacy of the other ligand and can enhance or impair signaling (Milligan, 2009). Another facet of heterodimerization is the creation of novel binding sites upon complex formation, which has been found for opioid receptor heteromers (Waldhoer et al, 2005).

Heteromers can induce a switch in G protein activation or coupling to induce distinct downstream signaling pathways. Additionally, they can also be implicated in biasing signaling and preferentially activate a specific downstream pathway (Rozenfeld & Devi, 2011).

Heteromerisation can also modulate surface delivery of receptors (Achour et al, 2008) by inducing a ER retention or inhibiting internalization (Jordan et al, 2001) (Cao et al, 2005).

*Physiological and pathophysiological relevance of heteromers*

The description of heteromer formation and function was mainly carried out by the use of cellular models, but more and more results prove its *in vivo* existence: $A_{2A}/D_2$ dimers can be found in the central nervous system (CNS) (Agnati et al, 2005) and a recent example proved evidence for melatonin receptor heteromers and their functional importance in the retina (Baba et al, 2013).

The detection of $5\text{-}HT_{2a}/GluR_2$ heteromers could provide targets for antipsychotic drugs that diminish hallucination symptoms in psychotic patients (Fribourg et al, 2011).

For the future, the discovery of more heteromer complexes with unique pharmacological properties can reveal novel therapeutic targets (van Rijn et al, 2013). The synthesis of peptides that interfere with heteromer formation (Rozenfeld & Devi, 2010; Rozenfeld & Devi, 2011) and selective or bivalent ligands for heteromers (Milligan, 2006) have enormous potential and might aim to provide new solutions for disorders which lack specific drugs.

## 1.5 Crosstalk of signal transduction pathways

In addition to their typical signal transduction pathways, GPCR signaling can cooperate with other signaling pathways, which is called signaling crosstalk. A crosstalk is the interplay between independent signal transduction pathways that influence or alter the outcome of one of the involved signaling pathways. A crosstalk can be achieved by (I) direct interaction of members of two different pathways, (II) indirect enzymatic or transcriptional action (III) competition events between signal transduction pathways (illustrated in Figure 8).

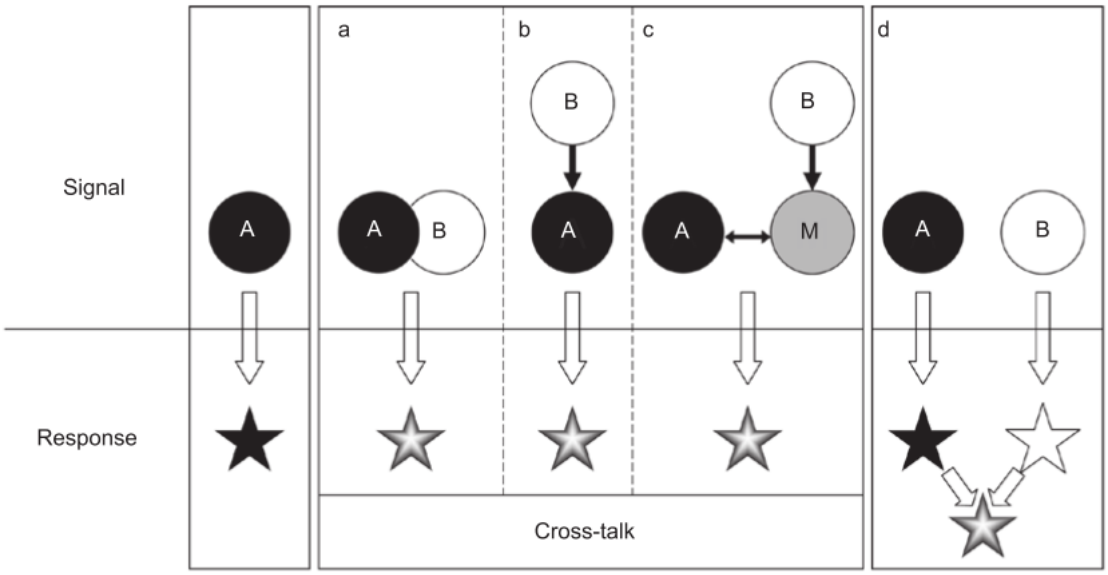



**Figure 8 Crosstalk in signal transduction (Guo & Wang, 2009)**

The figure explains the different possibilities of signal transduction pathways. Compared to the signaling in an independent pathway (left side), this signal can be influenced due to an interplay with other signaling pathways via (a) direct interaction, (b and c) one being an enzymatic or transcriptional target of the other in direct manner or through a mediator M or (d) two members of independent signal transduction pathways can compete on another protein.

### 1.5.1 Crosstalk between GPCRs

The crosstalk that occurs between GPCRs can have several origins. One source of crosstalk is the above-described formation of heteromers as described above: the physical interaction on the level of the plasma membrane often results in altered downstream signaling. This includes also the phenomenon of receptor transactivation from one to another (Vischer et al, 2011).

But the crosstalk between GPCRs can also take place in intracellular downstream signaling. GPCRs can, for example, compete for the same G protein subunits as it has been shown for

opioid and cannabinoid receptors, which are not assembled as heteromers but counteract on a later level of signaling (Canals & Milligan, 2008). Another example of crosstalk is the case of heterologous desensitization, which is the downstream activation of protein kinases, like the GRKs, by one receptor, which in turn phosphorylates another GPCR and induces their silencing (Chu et al, 2010).

Especially class C GPCRs serve as a good tool to study and understand the different mechanisms of inter-GPCR crosstalk (Prezeau et al, 2010) and the example of the $mGluR_{1a}$-$GABA_B$ crosstalk has been physiologically demonstrated in the CNS (Hirono et al, 2001).

### 1.5.2 Crosstalk with other signal transduction pathways

GPCRs are also able to interfere with and to be regulated by signal transduction pathways implicating other proteins than GPCRs.

*Crosstalk with receptor tyrosine kinases*

Already in the 90's it has been shown that a GPCRs can transactivate receptor tyrosine kinases (RTKs) in the absence of the RTK ligand. A well-described model is the transactivation of the EGFR by the lysophospholipid receptor LPA (Daub et al, 1997). The dissection of the mechanism showed that non-receptor tyrosine kinases like Src and Pyk function as intermediates through direct interaction with the RTK after their GPCR-mediated activation. Another mechanism could also pass in a ligand-dependent manner by activation of EGF ligand secretion through GPCR stimulation. New models like the triple-membrane-passing model propose the passage of the signal from the active GPCR over to metalloproteases, that act as intermediates for shedding of heparin-binding-EGF which then binds and activates its receptor (Fischer et al, 2006). The result of this transactivation is the formation of multimeric protein complexes, often with β-arrestins, that concertize in the regulation of MAP kinase activity.

Additionally, other GPCRs, such as LPA-, endothelin- and thrombin receptors can activate the platelet-derived growth factor (PDGF)-, the insulin-like growth-factor (IGF)- or the neurotrophin TrkA and B -receptors (listed in (Wetzker & Bohmer, 2003).

Conversely, RTKs can also transactivate GPCRs in the absence of their corresponding ligand, like the IGF receptor activates CXCR4 and the nerve-growth-factor (NGF) RTK activates the $LPA_1$ GPCR. This occurs mainly through direct interaction, but other mechanisms like ligand synthesis or transphosphorylation are possible (Delcourt et al, 2007a). A crosstalk between

the fibroblast growth factor (FGF) receptor and GPCRs has been demonstrated in several examples and different physiologic consequences: together with $D_2$ in schizophrenia, with $A_{2A}$ that has a role in synaptic plasticity (Flajolet et al., 2008) and with 5-$HT_{1A}$ in depression (Borroto-Escuela et al., 2011).
The crosstalk with RTKs explains the implication of GPCRs in growth control and vascular remodeling, which are not part of the variety of biological responses rising from independent GPCR signaling.

*Crosstalk with non-receptor tyrosine kinases*
Crosstalk can also occur with intracellular proteins. First evidence was provided by the chemical inhibition of soluble tyrosine kinases that led to a decrease in GPCR-mediated activation of MAP kinases, proposing their function as signaling intermediates. An involvement of Src kinase in GPCR-mediated activation of the MAP Kinases ERK, c-Jun N-terminal kinase (JNK) and p38 has been identified (Cao et al, 2000; Ma et al, 2000). Recent findings also propose a strong implication of GPCR bound β-arrestin mediating the activation of MAPKs. This suggests the creation of physical signaling platforms of RTKs and GPCRs and lead to the involvement of GPCRs in growth control *(Pyne & Pyne, 2011).*

*Crosstalk with PI3 kinase pathway*
GPCRs have also been found to transactivate PI3 kinases and their downstream signaling, either leading to Akt (Murga et al, 2000) or MAP Kinase (Lopez-Ilasaca et al, 1998) activation.

*Crosstalk with receptor serine/threonine kinases*
Compared to RTKs, only recent findings provide evidence for an extension of the transactivation to the RS/TKs receptors of the TGFβ signaling pathway (Burch et al, 2012), further described in chapter 3.4.

*Crosstalk with ion channels*
An interaction of the opioid-receptor-like GPCR ORL1 receptor can activate ligand gated calcium channels in absence of their ligand (Beedle et al, 2004).

*Implication of GPCR signaling elements in other signal transduction pathways*

In return, it has been described that RTKs use elements of GPCR signal transduction.

This is the case the IGF receptors that use the constitutive active CXCR12 GPCR, without its CCL12 ligand, to benefit from its $G\alpha_i$ activity in MDA-MD-231 cells (Akekawatchai et al, 2005). In order to activate adenylyl cyclases, the IGF receptor teams up with the pituitary adenylate cyclase-activating polypeptide PAC1 receptor signaling (Delcourt et al, 2007b). The physical interaction of the vascular endothelial growth factor (VEGF) receptor and the sphingosine1-phosphate receptor S1P1 also leads to a specific downstream signal transduction (Bergelin et al, 2010). The transactivation from TrkA to $LPA_1$ even seems to lead to nuclear translocation of the complex (Moughal et al, 2004). The existence and physiological relevance of the crosstalk has also been demonstrated in the CNS (Shah & Catt, 2004).

These different types of crosstalk add another brick in the multifunctionality concept of GPCRs and make them an important element of the cellular network, instead of functioning individually apart (Marinissen & Gutkind, 2001).

## 1.6 Orphan GPCRs

All these signal transduction pathways and crosstalks are mainly valid for the GPCRs with a known ligand. But there is still a part of proteins left that display sequence homology with GPCRs and have the same basic 7TM structure, but lack the identification of the corresponding natural ligand. The detection of increasing amounts of sequences of 7TM proteins without known proper ligand caused the definition of them as orphan receptors (Libert et al, 1991).

Today, we count in total more than 100 orphans, meaning 15% of all the GPCRs, and about one third by excluding the ~400 proteins of the olfactory fraction of GPCRs. Most of these orphans are members of the rhodopsin class A family (~ 80), nearly all of the adhesion GPCRs are still orphans (~30), and also 7 class C GPCRs, a secretin family member and some 20 taste receptors have currently no identified ligand (see Figure 8). A database that provides the up to date information about newly identified ligands can be found at http://www.iuphar-db.org.

These orphans build for two reasons an interesting object in research: (I) the identification of the matching ligand, their deorphanization, is still of big interest and (II) increasing evidence

exists for ligand-independent functions of orphan receptors. Therefore, orphan receptors are a source for the identification of further and formerly unappreciated functions of GPCRs.

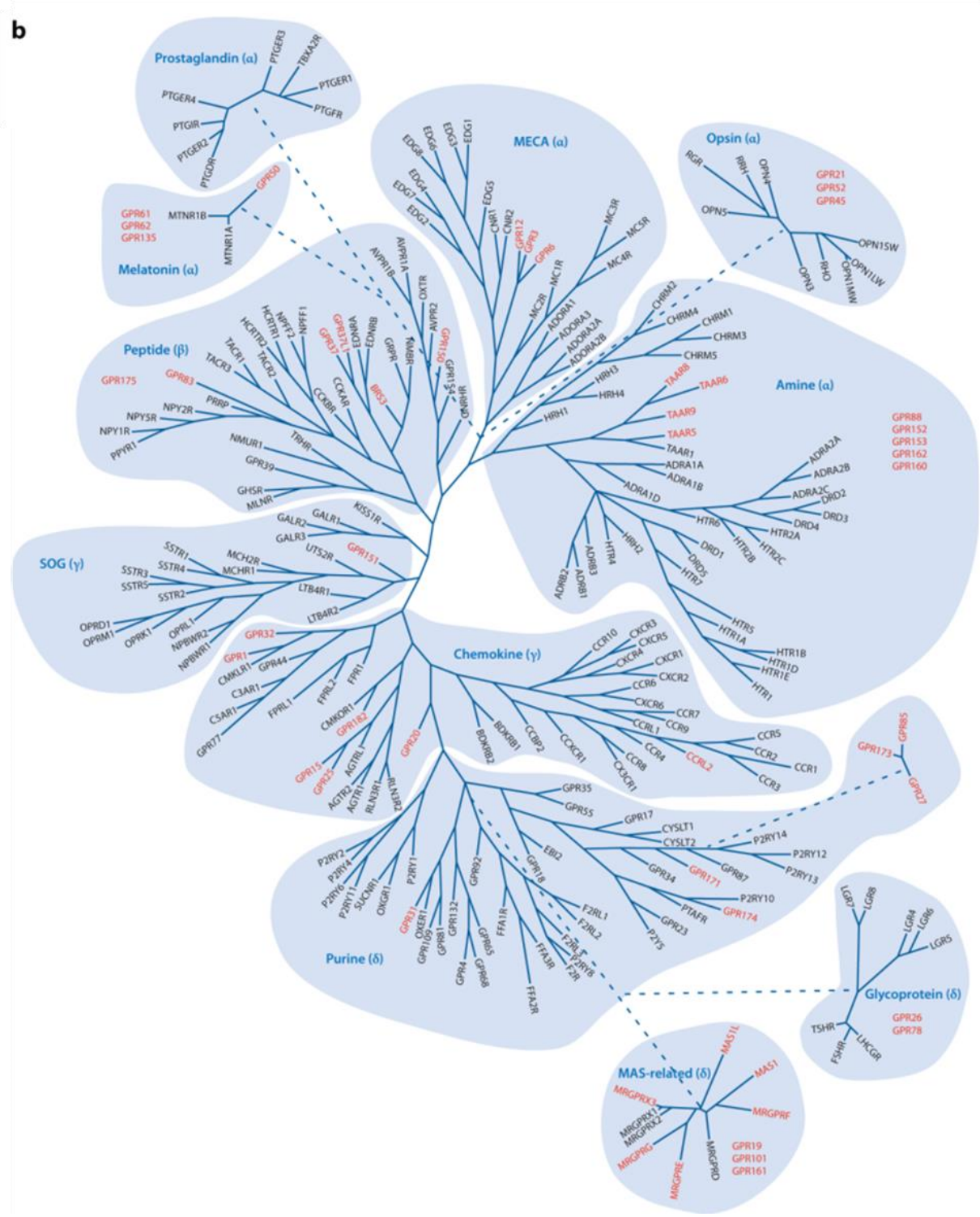



**Figure 8 Orphan GPCRs (Civelli et al, 2013)**
The figure shows orphan class A GPCRs according to their homology with existing subfamilies.

### 1.6.1 Deorphanization of GPCRs

A first aim for all the orphan receptors is to identify their natural ligand, the deorphanization. With the identification of other putative GPCRs due to their sequence homology and following cloning experiments in the early 90's (Marchese et al, 1994), a repertoire of orphan 7TM proteins was available. In order to identify suitable ligands, either *in silico* sequence alignment helped to find similarities to existing GPCRs or neurotransmitter molecule libraries were matched up with orphan GPCRs (Civelli et al, 2006).

*Reverse pharmacology*

The existence of such big collections of receptors without ligands initiated the development of new strategies in pharmacology. The approach from an available receptor towards ligand identification was termed reverse pharmacology (Mills & Duggan, 1994). It includes the heterologous expression of the orphan protein in cells, the incubation with a ligand from molecular libraries or tissue extracts and a subsequent analysis by functional assays. (Civelli, 2005).

The era of reverse pharmacology led successfully to receptor deorphanization but also the discovery of novel classes of ligand and receptors, like new neuromodulator families with nociceptin, orexins, prolactin-releasing peptide, ghrelin, apelin and kisspeptin (Civelli, 2012). Additionally, deorphanization could identify some lipid-like ligands and surprisingly previously unappreciated substances like uridine diphosphate (UDP)-glucose or citric acid cycle intermediates as GPCR ligands. Some receptors were found to be able to bind several different substances, like for the class C GPRC6A that binds different basic amino acids. This induced a shift away from the "one ligand-one receptor" dogma (Civelli et al, 2013).

*The post-reverse pharmacology age – new methods for deorphanization and future challenges*

Since 2005, we recognize stagnation in ligand identification, marked by a slower deorphanization rate that implemented the development of new approaches that include:

(1) *in silico* methods

to bioinformatically compare expression profiles of receptors and possible ligands and to use structure predictions of orphan proteins, which allow a forecast for the chemical structure of binding what in turn improves the generation of synthetic ligands.

(2) functional tests and their coupling to new read outs

One way to improve the sensitivity of established assays is the use of light-based methods as fluorescent labeling of receptors to assess their internalization, the fluorescence- (Ferguson & Caron, 2004) or luminescence- (Southern et al, 2013) based detection of β-arrestin recruitment or a combination of the measurement of cAMP production with fluorescence or luminescence. Additionally, the technique of reporter gene assay also made its way into GPCR deorphanization by the utilization of constructs with cAMP- or the PKC-activation-dependent transcription-factor-binding elements in their promoter (Yoshida et al, 2012).

(4) analysis of physiological function of GPCRs

The use of heterologous expression models (Sugita et al, 2013) or transgenic mice models with deletion of the protein of interest (Yang et al, 2013) can give idea about their function and physiological relevance and can in turn narrow down the number of ligands to be tested.

Beside the improvement of deorphanization methods, there are still some points that have to be paid attention to in the future in order to succeed: the discovery of increasing signaling possibilities and unpredictable G protein coupling of GPCRs make it more difficult to choose the appropriate method for deorphanization. It also has to be taken in account that heterologous expression models represent an artificial cellular environment and that either unnatural responses can be detected or additional interacting proteins, co-factors or heterodimerisation, which are required for activation, might be missing. The possibility that one orphan might only be activated by the simultaneous binding of more than one ligand could provide explanation for failure in deorphanization (Levoye & Jockers, 2008).

In contrast, for some GPCRs a ligand once has been identified, but it could never be proven again, as for GPR39 and obestatin binding (Civelli et al, 2013). In the case of GPR37, a first putative ligand, the neuropeptide head activator, has been identified in 2006 (Rezgaoui et al, 2006), what has never been repeated, while recent data provide another ligand possibility with the neuroprotective and glioprotective factors prosaptide and prosaposin (Meyer et al, 2013).

For the future, it will be important to adapt the current state of knowledge about GPCRs and their signaling, including all the new concepts and findings, for the deorphanization of orphan GPCRs and to develop new techniques and strategies for their ligand identification.

### 1.6.2 Ligand-independent functions of orphan GPCRs

Even though, Civelli hypothesized that the rules of evolution implicate the existence of a ligand for every GPCR (Civelli, 1998), combined with the difficulty to imagine the evolutionary survival of receptor-like proteins without ligand, increasing evidence proposes a ligand-independent function for GPCRs. For these proteins, the term "orphan 7TM protein" seems to be more suitable, because their sequence predicts the existence of the heptahelical core, but their coupling to G proteins is not obligatory.

The characterization of orphan 7TM proteins started with the development of cloning techniques in the beginning of the 90's that enabled overexpression in cells and founds its continuation in methods using genetic ablation in cells or transgenic mice, permitting the identification of functions and modes of action for the 7TM proteins.

*Constitutive activity*

One possibility for the orphan GPCRs is the case of constitutive activity. This concept has been proven for virus encoded 7TM proteins like UL33 (Waldhoer et al, 2002) and the Epstein-Barr virus-induced G-protein coupled receptor EBI2 (Rosenkilde et al, 2006) and for non-viral 7TM proteins like GPR3 (Ledent et al, 2005; Mehlmann et al, 2004), GPR6 and GPR12 (Tanaka et al, 2007), GPR26 and GPR78 (Jones et al, 2007) that constitutively activate adenylate cyclase. But it remains under speculation whether this could be an artificial effect upon overexpression or the result of permanent occupancy with the endogenous ligand as it is the case for the fatty acid receptor GPR40 (Stoddart et al, 2007). Whether other ligand-independent mechanisms exist to regulate their activity or to inhibit their constitutive activity is unknown, but constitutive activity of orphan GPCRs can be applied for the design of suitable inverse agonists.

*Modulation of the function of other proteins upon direct interaction*

Another area for the characterization of the function of orphan 7TM proteins lies in their ability to interact with other proteins. The utilization of proteomic approaches can help to identify putative binding partners of 7TM proteins. Recently, a lot of techniques specific for transmembrane proteins have been developed (Daulat et al, 2013), among them the tandem affinity purification (TAP) that allows the isolation under native conditions (Daulat et al, 2007). Their application to orphan GPCRs and other techniques helped to uncover protein complexes.

(1) Obligatory formation of heteromers with other GPCRs

A first role which has been attributed to orphan 7TM proteins, is heteromerisation with other GPCRs, which mainly occurs in members of the same subfamily. In some cases the interaction of an orphan protein can be absolutely required for the activation of the other protein. A well-described system is the one of the $GABA_B$ receptors. Heterodimerisation between $GABA_{B1}$ and $GABA_{B2}$ is mandatory for activation by the GABA ligand. Both proteins must be in complex to be able to transduce a signal, since each one has a specific function: $GABA_{B1}$ binds the ligand and $GABA_{B2}$ is responsible for transducing the signal to the G protein. In this case $GABA_{B2}$, who has no ligand binding domain, functions as the orphan receptor (Kniazeff et al, 2002).

(2) Activity-modulating, inducible heterodimerisation with other GPCRs

Another possibility is conditional heteromerisation of GPCRs, where the formation of heteromers can alter the function of the homomer. Various examples with different modes of action have been discovered in the last years. The pioneer work in this field demonstrated the heteromer formation of the melatonin receptor $MT_1$ and its orphan family mate GPR50. In this case, the orphan GPR50 negatively interferes with melatonin-dependent signal transduction (Levoye et al, 2006a) (for more details see Chapter 2).

The β-alanine binding mas related receptor $Mrg_D$ can form a complex with its orphan relate $Mrg_E$. This interaction is associated with potentiation of signaling and inhibition of internalization of the receptor (Milasta et al, 2006).

But there are also some examples which demonstrate a heteromer formation between non-homologous proteins. This can even occur between the 7TM proteins of different species. The orphan receptors UL33 and UL78 from the human cytomegalovirus are able to interact with the chemokine receptors CCR5 and CXCR4 of their host cells and modulate their function (Tadagaki et al, 2012). Another example for an interaction of proteins of different species has been shown with EBI that binds to CXCR5 (Barroso et al, 2012).

An interesting example is the one of a conditional orphan receptor. A protein with a known ligand can behave under certain circumstances as orphan receptor. This was recently demonstrated for the ghrelin receptor $GHSR_{1a}$. In hypothalamic neurons, where ghrelin is not present, the receptor forms heteromers with the $DRD_2$ dopamine receptor and modulates its activity, which induces the anorexigenic properties of dopamine stimulation. The independence of this interaction of the ghrelin ligand was additionally demonstrated with the use of ghrelin KO mice (Kern et al, 2012).

(3) Association with other proteins

So far, only few examples describe the interaction of orphan 7TM proteins with other proteins than GPCRs. The long-time orphan GPR37 has been shown to interact with the dopamine transporter DAT to modulate dopamine uptake (Marazziti et al, 2007). The orphan GPR50 can interact with neurite-outgrowth-inhibitor Nogo-A (Grünewald et al, 2009) and the transcription factor TIP60 (Li et al, 2011), described later in chapter 2.3.3.3. Another example is the inhibition of the constitutive activity of the frizzled class GPCR Smoothened by the hedgehog ligand. Hedgehog binds via the 12TM protein Patched to smoothened, and Patched itself is thereby responsible for inhibiting constitutive activity of Smoothened (Riobo et al, 2006).

*Other functions of orphan 7TM proteins*

Another axe of research is the identification of functions independent of protein-interaction for orphans. So far, only little data exist giving information about other potential actions of orphan 7TMs proteins. One example is nuclear translocation, either partial or total, which has been shown for GPR50 (Li et al, 2011) and GPR158 (Patel et al, 2013) respectively.

## 1.7 GPCRs as drug targets

A large part of the available pharmaceuticals target GPCR activity and for about 15 to 30% of each molecular class of ligand, appropriate drugs have been developed, most of them for receptors of biogenic amines.

The emerging knowledge about the functionality and diversity of GPCR signaling will allow the development of more various and specific drugs at once. Promising possibilities arise from biased signaling (Kenakin & Christopoulos, 2013) and allosterism (Valant et al, 2012) to design more specific and selective drugs in order to better target the diseases and avoid undesired side effects. Additionally, the recent concept of GPCR oligo- and heteromerisation can also lead to a development of new classes of curing substances like bivalent ligands or substances that interfere with complex formation (Allen & Roth, 2011). Emerging *in silico* use, can help to improve the generation of synthetic drugs, where a design suitable for orphan receptors can also be imagined.

In combination with modern techniques of industry, drug design for GPCRs will reach another dimension by shifting from general treatment for a GPCR to one that is adapted to target the condition-specific signaling of each receptor.

# 2. The GPCR subfamily of melatonin receptors and GPR50

$MT_1$ and $MT_2$ receptors for the amine melatonin and the orphan 7TM protein GPR50 form the melatonin receptor subfamily of the class A rhodopsin-like GPCRs.

## 2.1 Melatonin synthesis and function

The origins of melatonin (5-methoxy-N-acetyltryptamine) go back to 1917, when Allen and McCord described that extracts from bovine pineal glands can bleach tadpole skin. Then, in 1958, Lerner and colleagues successfully isolated melatonin from bovine pineal glands (Lerner et al, 1958).

Melatonin is synthesized during the night primarily by the pineal gland, but also, although to a lesser extent, by tissues like retina, intestinal organs, skin, blood cells and lymphocytes. The amino acid tryptophan is the precursor of melatonin (see Figure 9) (Klein et al, 1997). Pineal melatonin synthesis is regulated by light and the circadian rhythm generated by the hypothalamic suprachiasmatic nucleus (SCN).

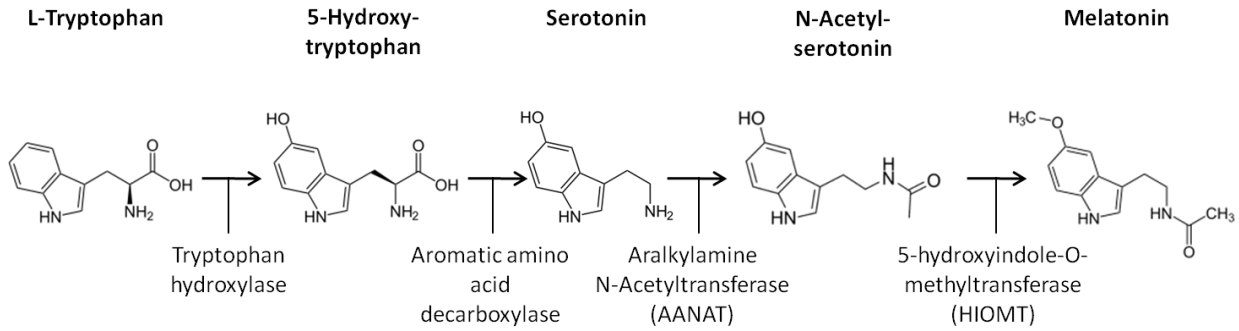



**Figure 9 Melatonin synthesis**

Melatonin is synthesized out of the essential amino acid L-tryptophan. After enzymatic hydroxylation and decarboxylation, serotonin is formed. This intermediate then gets transformed through enzymatic addition of an acetyl group by the AANAT, which is the limiting step during melatonin synthesis. The HIOMT then transfers a methyl residue, which forms melatonin out of N-Acetylserotonin.

Melatonin is a lipophilic molecule, which can easily cross the blood-brain barrier and circulate through the bloodstream to reach peripheral organs. Its half-life is about 10 minutes before it undergoes cytochrome-mediated hepatic transformation into hydroxymelatonin prior to excretion (Vanecek, 1998).

One of the main tasks of melatonin is the synchronization of circadian rhythms and the regulation of reproduction in seasonal animals. The other actions of melatonin in the brain also explain its implication in psychiatric and neurological disorders. It plays a role in depression, sleep and seasonal affective disorders and jetlag-related effects. An association also seems to exist with Alzheimer disease in melatonin decelerating Alzheimer progression (Savaskan et al, 2007; Savaskan et al, 2001). Beside its function in the brain, the melatonin signal reaches tissues either through their neuronal connection to the SCN, or in a humoral manner of melatonin circulating in blood and binding to its corresponding receptors on different organs (Hardeland et al, 2011). In the periphery, melatonin regulates functions of the cardiovascular and the immune system (Calvo et al, 2013; Markus et al, 2007), metabolism and glucose homeostasis (Karamitri et al, 2013).

## 2.2 The family of melatonin receptors

The effects of melatonin are mediated by its corresponding receptors present at the cellular plasma membrane. The discovery of melatonin receptors became possible with the development of radioactively labeled 2-iodomelatonine in 1984 (Vakkuri et al, 1984a; Vakkuri et al, 1984b). The radioligand enabled the localization and detection of melatonin receptors in different tissues.

First attempts to clone the melatonin receptor occurred in the first half of the 90's and the first receptor to be cloned was $Mel_{1c}$ from Xenopus (Ebisawa et al, 1994). Cloning of the mammalian receptors followed in the next years (Reppert et al, 1995; Reppert et al, 1994). The subfamily of melatonin receptors is composed of three family members, $MT_1$ and $MT_2$ and $Mel_{1c}$ the latter only existing in vertebrates (fish, amphibia, birds and reptiles) and having evolved into the orphan 7TM protein GPR50 in mammals. $MT_1$ is expressed in every species, while $MT_2$ is not found in hamster. Another melatonin binding protein, called $MT_3$, corresponds to the quinone reductase $QR_2$ (Nosjean et al, 2000; Nosjean et al, 2001). Compared to $MT_1$ and $MT_2$, $MT_3$ binds melatonin with lower affinity and is structurally very different. $MT_3$ might be responsible for the implication of melatonin in detoxification and possibly accounts for some of melatonin's antioxidant effects.

*Genetics of melatonin receptors*

The genes for melatonin receptors are composed of two exons separated by one intron and are located on different chromosomes for $MT_1$ and $MT_2$. Several studies identified different

isoforms or polymorphisms in intronic and exonic regions of the corresponding genes that can slightly affect signaling, as demonstrated for $Mel_{1c}$ isoforms (Jockers et al, 1997). Recent studies have shown that rare exon variants of $MT_2$ are associated with an increased risk to develop type 2 diabetes (Bonnefond et al, 2012).

*Structural features of melatonin receptors*

The three melatonin receptors $MT_1$, $MT_2$ and $Mel_{1c}$ share a sequence homology of 60% that increases up to 73% in the TM region (Gubitz & Reppert, 2000). They are about 350 amino acids longs and have a molecular weight of 40 kDa.

Sequence analysis allowed their classification as rhodopsin-like receptors. They share some common features with other class A GPCRs like the existence of the N-terminal glycosylation sites, cysteine residues in ECLs to form disulfide bonds with the 7TM core, phosphorylation sites and palmitoylation sites in the C-terminal part of the receptor. Distinctive structural features only found in the melatonin receptor subfamily are the presence of the NRY motif instead of the E/DRY motif in TM3 and the NAXXY motif instead of the NPXXY motif in TM7.

*Tissue distribution and signal transduction of $MT_1$ and $MT_2$*

Melatonin receptors generally display low expression levels. By lacking a good antibody, information about expression of the receptors was obtained mainly from *in situ* hybridization and radioligand binding studies. $MT_1$ is more abundantly expressed than $MT_2$. The highest amounts of $MT_1$ are found in the pars tuberalis, SCN and retina, and it is also expressed in hippocampus, cortex and the tanycytes (von Gall et al, 2002). Furthermore, the receptors are found in many of the peripheral tissues. $MT_2$ displays similar expression patterns with lower levels, but it still remains challenging to distinguish between both receptors.

*Signal transduction of melatonin receptors*

The studies to decipher melatonin receptor function were carried out in tissue with elevated MT receptor expression or appropriate cellular models. It was proven, that melatonin receptors signal via $G\alpha_i$ proteins by the decrease of cAMP due to inhibition of the adenylate cyclase and its sensitivity to Pertussis toxin (Vanecek & Vollrath, 1989). The $G\alpha_i$ downstream activity has been shown with inhibition of PKA activity and the lack of CREB phosphorylation (Witt-Enderby et al, 2003). Additionally, melatonin can inhibit cGMP

production via $MT_2$ (Petit et al, 1999). Other signaling pathways that are activated upon Melatonin stimulation implicate $G\alpha_q$ coupling and Gβγ-dependent PLC activation with subsequent IP3 production and calcium release from internal stores. Also the coupling to β-arrestins and subsequent ERK activation has been demonstrated (Kamal et al, 2009).

*Receptor heteromerisation*

A co-expression of $MT_1$ and $MT_2$ in several tissues led to the suggestion, that they might form heterodimers. Utilization of the BRET technique (Ayoub et al, 2002) helped for to the successful detection of homo- and heteromeric structures with a preferential formation of $MT_1/MT_2$ or $MT_1/MT_1$ dimeric structures (Ayoub et al, 2004). Recently, the occurrence and relevance of heteromers in retinal physiology was demonstrated *in vivo* (Baba et al, 2013).
Another aspect is the heteromerisation with the orphan family member GPR50 that negatively influences melatonin binding and signaling, which will be discussed in detail below.

## 2.3 The orphan 7TM protein GPR50

The subfamily of melatonin receptors contains another member, which for the first time appeared in rodents during evolution, the orphan 7TM protein GPR50. It was discovered by screening a bank of human pituitary cDNA with degenerated primers in order to amplify proteins with high sequence homology to $MT_1$ and $MT_2$. The protein of 613 amino acids was named melatonin-related-receptor, that later became GPR50 (Reppert et al, 1996).

### 2.3.1 Origins and structure of GPR50

*Phylogenetic origins of GPR50*

GPR50 is a protein that is exclusively found in mammals and for long time, its evolutionary origins remained unclear. Sequence analysis revealed, that it developed out of the $Mel_{1c}$ melatonin receptor, which is only found in lower metazoa as reptilia, fish and chicken. The finding that GPR50 is the mammalian ortholog of $Mel_{1c}$ was unexpected because of the low percentage of sequence homology. Further analysis could show that GPR50, compared to $Mel_{1c}$, underwent translocation from chromosome 4 to chromosome X and fusion with another gene, that is supposed to be an ancestor of the RNA polymerase II (Dufourny et al, 2008). Sequence comparison of $Mel_{1c}$ and GPR50 in the 7TM part revealed, beside the amino acid changes that are the result of evolutionary pressure that a surprisingly high amount of

neutral evolution of 28% occurs in GPR50. One might speculate, that these relaxed substitutions occurred probably because melatonin binding to $Mel_{1c}$ was not required anymore in higher species, diminishing the pressure for amino acid conservation or directed exchange (Tian et al, 2009).

A comparison between the sequences of the different species, which contain the gene for GPR50 shows that its sequence is less conserved among different species compared to the other melatonin receptors. While the 7TM part exhibits about 90% sequence homology between mouse, sheep and humans, the cytosolic region has more evolved. For example, the 591aa long mouse GPR50 (Gubitz & Reppert, 1999a) and the 575aa long sheep GPR50 (Drew et al, 1998) both share only 74% homology in total with the human sequence.

*Genetic and protein structure of human GPR50*

The GPR50 gene is organized like the other melatonin receptors and composed of two exons separated by one intron. Exceptions are found in horse, where exon 1 is splitted into four exons and in opossum, where it is composed of 7 smaller exons. In the 3' region of the GPR50 gene, an important region for the regulation of gene transcription, a consensus site for a specific micro RNA, the has-mir185 has been recognized (John et al, 2004). This miRNA is implicated in regulation of circadian genes like cryptochrome1 (Cry1) (Lee et al, 2013) and might probably be related to cancer by inhibiting tumor growth (Takahashi et al, 2009; Yoon et al, 2013). Additionally, the identification of CpG islands that are targets of DNA methylation, in the intronic region, propose epigenetic regulation mechanisms for GPR50 gene expression. Indeed transcription of GPR50 is regulated by the DNA methyltransferase Dnmt3a1 (Kotini et al, 2011). The human gene product of GPR50 has a size of 67 kDa and displays in total 45% homology with $MT_1$ and $MT_2$. In the hydrophobic transmembrane core part, it rises up to 55% (see Figure 10).

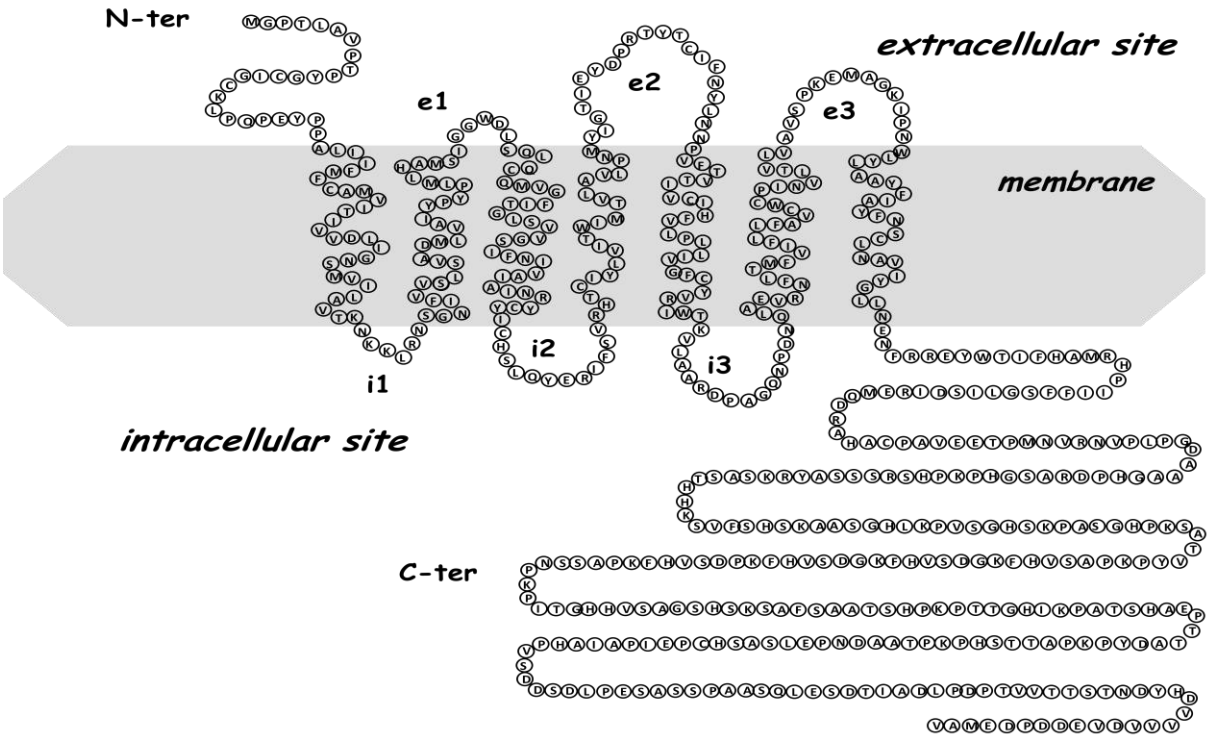



**Figure 10 Structure and topology of GPR50**

GPR50 is composed of a heptahelical core with extracellular and intracellular loops and a characteristic C-terminus. It shares common features that are unique for the subfamily of melatonin receptors as the NRY motif in the ICL2 and the NAVIY motif. The C-tail is 318 aa long and derived from gene fusion with an ancestor of RNA polymerase II.

The comparison to the melatonin receptor structure shows common characteristics as cysteine residues to form a disulfide bridge between ECL2 and ECL3, the NRY motif in TM3 and the NAVIY sequence in TM7. But some features are also altered or missing. GPR50 has no N-glycosylation site in the N-Terminus and the ECLs (Barrett et al, 2003).

*Long C-terminal region*

GPR50 has a 318 amino acid long cytoplasmatic C-terminal tail, which is much longer than the typical size of class A GPCRs of 20 to 90aa. This part is the product of genetic fusion with an ancient RNA polymerase II. It contains a highly repetitive sequence of several degenerated heptapeptides (see Figure 11):

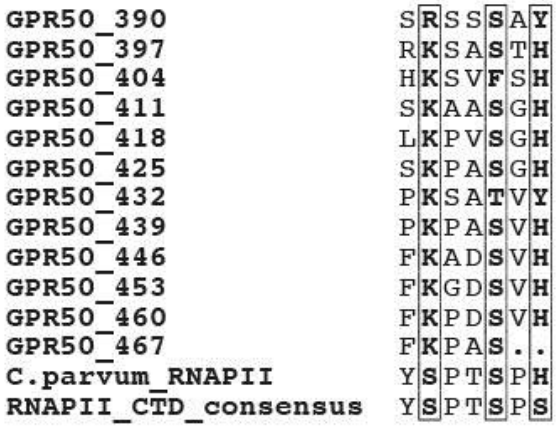



**Figure 11 Heptapeptid repeats in the C-terminus of human GPR50 (Dufourny et al, 2008)**

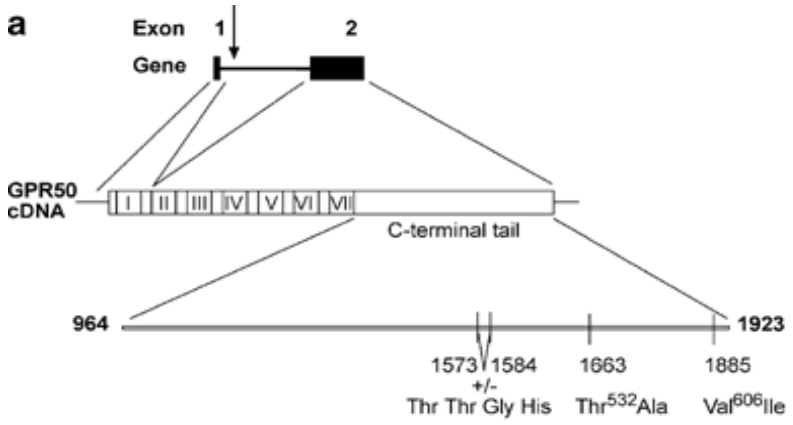
The C-terminal part of human GPR50 presents a repetitive heptapeptid structure: in the first and last position is an aromatic amino acid, the sixth is aliphatic and hydrophobic one, the second is a basic lysine and the fifth one with a hydroxyl-residue like Ser. Below are represented consensus repetitive sequences of the RNA Pol II, the gene of origin of GPR50, displaying similar amino acids.

Furthermore, SH dipeptides in the distal region might be putative phosphorylation sites. Especially this repetitive part is strongly resembling the C-terminal repetitive domain of RNA Pol II, that functions as scaffold for factors that regulate transcription in a phosphorylation-dependent manner (Dufourny et al, 2008). These features lead to the hypothesis that this part of GPR50 might be subject to phosphorylation and form a scaffold for other proteins

*Existence of sequence variants*

A first targeted gene approach carried out by Thomson in 2005 (Thomson et al, 2005) revealed the existence of sequence polymorphisms in GPR50. Three of them in the coding region of the exons: one insertion/deletion of 12 base pairs that encode for the four amino acids Thr-Thr-Gly-His at position 502 to 505 TTGH, a Thr>Ala exchange at position 532 that is in complete linkage disequilibrium with the deletion. At position 606 we find another substitution of Val>Ile that occurs independently from the other ones (see Figure 12).

These polymorphisms occur with a high frequency of about 40% in the population, consequently one prefers to speak of sequence variants instead of polymorphisms, that usually only account for 1% or less. Another consequence is the denomination of the insertion variant with 617aa as GPR50 wildtype, while the other, originally cloned version of 613aa, is the mutant form of GPR50.




b

| Variant Name | Nucleotide change |
|---|---|
| Δ502-505 | Deletion of 1573 → 1584 inclusive (ACC ACT GGC CAC) |
| $Thr^{532}Ala$ | 1663 A → G |
| $Val^{606}Ile$ | 1885 G → A |

**Figure 12 Localization of polymorphisms in the exons of GPR50 (Thomson et al, 2005)**

The GPR50 can occur in different sequence variants of the exon 2: (I) A 12 base pair deletion, that leads to deletion of the TTGH amino acids from position 502 to 505 and is always accompanied by (II) an Thr>Ala exchange rising from one A to G base substitution. (III) Another sequence variant is the replacement of a G by an A that changes Val to Ile. All polymorphisms occur with about 40% in the population, classifying them as sequence variants.

### 2.3.2 Expression pattern and tissue distribution of GPR50

An interesting question is, whether GPR50 displays similar expression pattern as the melatonin receptors or if the evolution also led to an altered tissular distribution. First analysis started with mRNA *in situ* hybridization, northern blot and tissue reverse transcriptase (RT)-PCR based on mRNA levels. Only later, with the development of antibodies against GPR50 (Hamouda et al, 2007a) a characterization of protein localization was performed.

*GPR50 expression in the brain*

First studies concentrated on the localization of GPR50 mRNA in the brain. It was found in the pituitary and the mediobasal hypothalamus (ventromedial, paraventricular and arcate nucleus) which is overlapping with expression of $MT_1$ and $MT_2$ (Reppert et al, 1996). Additionally, a lot of cells around the 3rd ventricle, the tanycytes contain GPR50 mRNA. Sheep studies showed additional expression in retina, pars distalis and pars tuberalis of the pituitary, the region coordinating reproduction (Drew et al, 1998).

Later studies by RT-PCR and *in situ* hybridization in rodents detected GPR50 in additional regions like the amygdale, the chorioid plexus, the subfornical organ, the preoptic area, the

bed nucleus of stria terminalis, the olfactory bulb, the parabrachial nuclei and the vascular organ of lamina terminalis (Drew et al, 2001).

Later studies using the first GPR50 specific antibody (Hamouda et al, 2007b), allowed to detect regions of GPR50 protein expression. Analysis by immunofluorescence could confirm strong expression of GPR50 in the dorsomedial hypothalamus (DMH), tanycytes and the median eminence (Sidibe et al, 2010). A later study showed that GPR50 is among the 6 highest genes expressed in the DMH (Lee et al, 2012), a region with an important role in energy homeostasis under circadian regulation. In the human brain, GPR50 was found also in the CA4 region of the dentate nucleus of the hippocampus (Hamouda et al, 2007b). The use of another antibody provided precise data for mouse, rat and sheep brain, that confirmed existing data obtained from mRNA expression studies (Batailler et al, 2011).

A recent study also investigated the expression of GPR50 in developing and adult mouse brain, that was based on the findings that GPR50 holds a role in neurite-outgrowth regulation (Grünewald et al, 2009). It was found that GPR50 is expressed throughout all developmental stages with peaks at E18, a late stage in embryonic development with axon formation. Furthermore, this study could identify new regions of GPR50 expression, that are involved in neurotransmission, as monoaminergic neurons (Grunewald et al, 2012).

*Regulation of GPR50 expression in the brain*

In the seasonal Siberian hamster a down-regulation of GPR50 expression was detected in short-day periods in cells of the ependymal layer of the 3$^{rd}$ ventricle, proposing a regulation by photoperiod (Barrett et al, 2006). A later study in a transgenic mouse where the lacZ gene replaced the GPR50 gene, showed that GPR50 expression in the 3$^{rd}$ ventricle is decreased upon high fat diet or fasting (Ivanova et al, 2008). Both studies suggest a regulation of GPR50 expression dependent on environmental and metabolic circumstances.

*Expression in peripheral tissues*

Expression levels of GPR50 in peripheral tissue were barely investigated. One study could reveal by RT-PCR that eye, testis, kidney, adrenal, intestine, lung, heart, ovary and skin express GPR50 mRNA (Drew et al, 2001). Most of these data could be confirmed by another PCR analysis (Li et al, 2011). For the future, the detection of GPR50 protein level in peripheral tissue will hopefully be achieved to gain information about possible functions outside the brain.

### 2.3.3 Physiological importance and function of GPR50

Identifying the function of an orphan receptor is always challenging. As a first approach the expression pattern of the protein can already give some hints about its function. These data suggest for GPR50 a strong implication in neuroendocrine functions, like food intake, energy homeostasis, thermoregulation, behavior and reproduction. In addition, different strategies detailed below have profitably helped to find out about different functions of GPR50 during the last years.

#### 2.3.3.1 The molecular basis of GPR50 being an orphan 7TM protein

After the successful cloning of GPR50, studies to test melatonin binding for the melatonin-related-receptor in COS cells remained negative (Reppert et al, 1996). This result could be confirmed by other groups for the mouse GPR50 (Gubitz & Reppert, 1999a) and the sheep protein (Drew et al, 1998). Also other screening assays by reverse pharmacology did not result in finding a cognate ligand for GPR50, and still nowadays modern techniques failed (Southern et al, 2013). Therefore, GPR50 currently seems to be a genuine orphan 7TM protein.

After having established the absence of melatonin binding in GPR50, further studies tried to identify the structural differences between melatonin receptors and GPR50. The construction of chimeric proteins helped to reveal the regions and amino acids, which are important for high-affinity melatonin binding and which are altered in GPR50. It was shown that especially TM6 of GPR50, notably the Gly257Thr- and some other amino acid substitutions seem critical for losing melatonin high-affinity binding (Conway et al, 2000; Gubitz & Reppert, 2000), as illustrated in Figure 13. Since high-affinity binding depends on the binding of the ligand and the G protein to the receptor, it still remains unclear whether these residues are involved in melatonin binding or in receptor activation leading to G protein engagement.

Later sequence analysis and comparison of amino acids between GPR50 and its $Mel1_c$ ancestor revealed amino acids that have positively evolved (see Figure 13). Their juxtaposition to the extracellular side indicates that they might be involved in a gating function, necessary to permit melatonin access to the ligand binding pocket inside the 7TM region. Probably, the evolution of these gating amino acids in GPR50 explains its loss of melatonin binding. Experimental validation of these predictions and the importance of sites in the ECL loops, especially ECL2 is still in progress (Clément, Guillaume & Jockers, unpublished data).

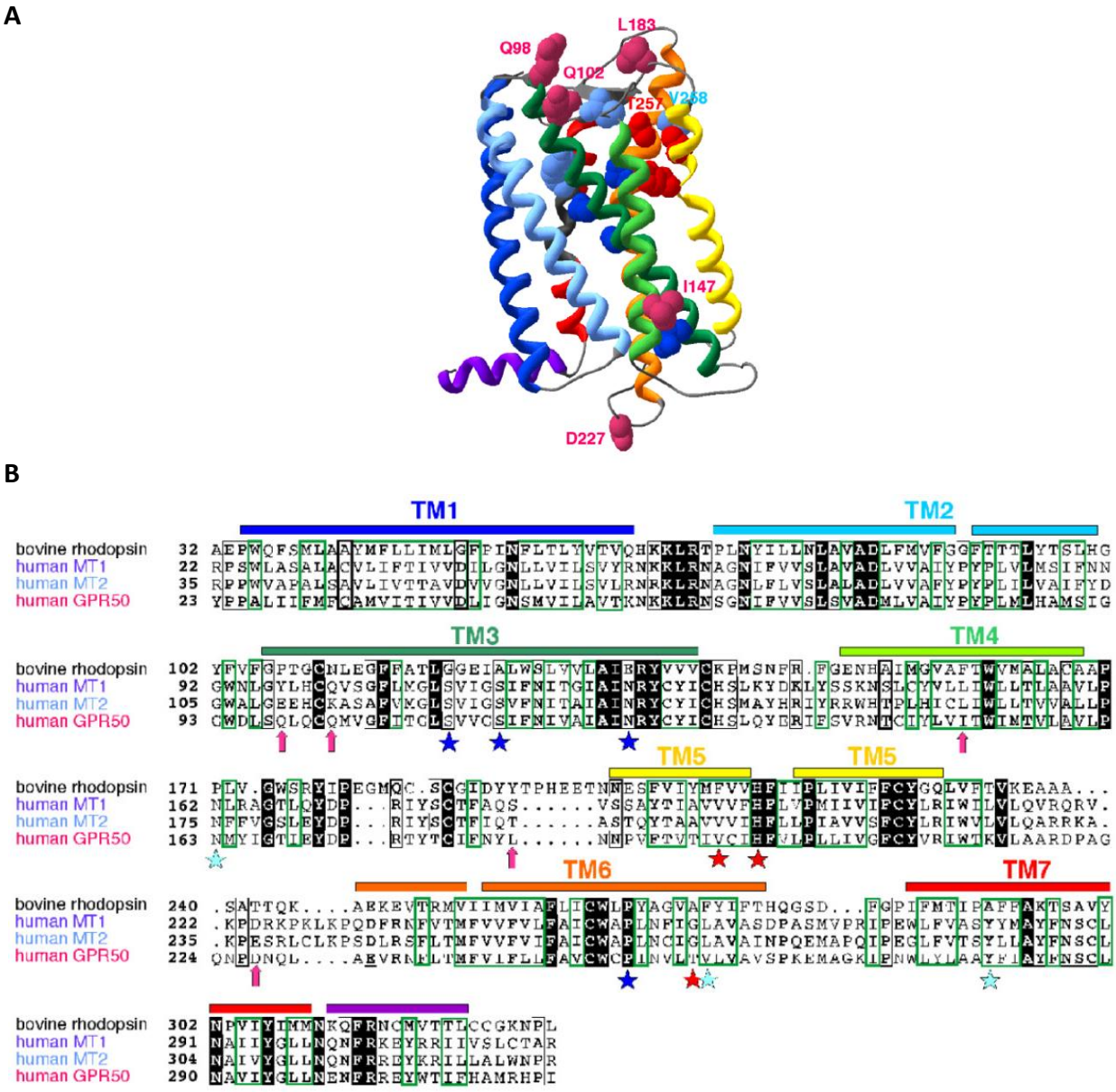



**Figure 13 Structural prediction of GPR50 and sequence alignment with melatonin receptors (Dufourny et al, 2008)**

(A) A structure prediction of GPR50 that highlights that the amino acids changes compared to the melatonin receptor. Most of them are located in TM6, as Thr257 (red) and Val258 (light blue). The analysis of amino acids that underwent positive evolutionary pressure (pink) are mainly localized in regions and face the extracellular site.
(B) The sequence alignment reveals amino acids that are important for melatonin binding in $MT_1$ (dark blue stars), $MT_2$ (light blue stars) or both (red stars). Comparison with the GPR50 sequence reveals some amino acids, which are changed and might account for the loss of melatonin binding ability in GPR50.

#### 2.3.3.2 Physiological role of GPR50

To determine the physiological impact of GPR50, several research axes and strategies can help to shed light on this question. Transgenic mice with deleted or non-functional GPR50 can serve to reveal an altered phenotype. Furthermore, genetic association studies with GPR50 variants or SNPs can also give us information about its physiological importance.

*Expression – function relation of GPR50*

A first idea about the function of GPR50 can arise from its expression pattern. The hypothalamic DMH is an important area of the brain to regulate food intake: it has a circadian clock that is food entrainable and additionally, it has an important role in stress responses. Another region with strong GPR50 expression are the tanycytes surrounding the 3$^{rd}$ ventricle. They form the connection between the cerebrospinal fluid (CSF) and the pituitary. Hence, these cells are important in sensing molecule concentration (like glucose) in the CSF, responsible for molecule transport from the CSF into the brain and they govern release of hypothalamic hormones. Additionally, tanycytes are part of the hypothalamus-pituitary-thyroid axe, and express a deiodinase for thyroid hormone activation.

G*enetic association studies of GPR50 polymorphisms*

The region of GPR50 localization on the X chromosome, Xq28, is associated with some neuroendocrine disorders (Dufourny et al, 2008), which is also supported by GPR50's expression pattern. The findings of the existence of GPR50 sequence variants opened the gate for genetic association studies of different disorders.

(1) Mental disorders

The first study in 2005 established a correlation of GPR50 polymorphisms with different mental disorders, as bipolar affective disorder (BPAD), major depression disorder (MDD) and schizophrenia in a Scottish population (Thomson et al., 2005). The mutant ΔTTGH variant holds a higher risk for BPAD and MDD, especially in females. A later study with further Scottish subjects also demonstrated an association in females with two ΔTTGH alleles with age of onset, increase in episode number and hypomanic periods (Macintyre et al, 2010).

Another intronic polymorphism, rs2072621 is associated with schizophrenia development in females. The strong sex specific component could be underlined by the observation of additive effects of two identical alleles, even though one X chromosome possibly undergoes inactivation. Later studies had difficulties to repeat the association in a Swedish cohort (Alaerts et al, 2006; Thomson et al, 2005), but the findings for association of the rs2072621

intron polymorphism in a French population (Delavest et al, 2012) and another intronic rs1202874 variant in Scottish people (Macintyre et al, 2010) with seasonal affective disorder (SAD) suggest a correlation between GPR50 function and the neuronal activities underlying these disorders.

A tendency for an association of the deletion variant with autism spectrum disorder was also be shown but did not reach statistical significance (Chaste et al, 2010).

With regards to neurological disorders, an increase of GPR50 immunoreactivity was found in the brain of Alzheimer patients (Hamouda et al, 2007a).

(2) Lipid metabolism

The results obtained from KO mice, that GPR50 is an important player in energy homeostasis got further support by genetic association studies. Even though, no differences in body mass index were observed, higher levels of fasting triglycerides in the blood of subjects with homozygous alleles of either intronic, ΔTTGH or V>I GPR50 variants (Bhattacharyya et al, 2006).

*Transgenic Knock-Out mice*

A preferential tool to study the physiological role of a protein consists in the utilization of mice where the gene of interest is deleted. The first KO mice for GPR50 was described in 2006 (Barrett et al, 2006). This mouse has a GPR50 gene that is interrupted through insertion of the LacZ gene, leading to expression of LacZ, which can be visualized, instead of GPR50. This mouse has been studied extensively with regards to metabolism (Ivanova et al, 2008). Mice lacking functional GPR50 show lower body weight, resistance to weight gain after high energy diet and less fat mass development while higher food intake, which makes them have a lower energy efficiency but a higher metabolic rate. Surprisingly, the GPR50 KO mice lose less weight under fasting conditions. In addition they display a tendency for hyperactivity with increase in oxygen consumption and $CO_2$ production. This is also supported by higher corticosterone concentration in the blood that might reflect higher stress levels. Unfortunately, this study could not clarify whether this hyperactivity is the reason for higher metabolic rate or whether less weight gain enables for higher activity. Additional expression analysis of the GPR50-LacZ protein revealed that GPR50 levels adapt to the energy status: both, high fat diet and fasting conditions lead to decrease in GPR50 expression. Taken together, these results suggest a role of GPR50 in energy homeostasis. Another study with KO mice (Bechtold et al, 2012) revealed the implication of GPR50 in torpor, a possibility for the body to cope with

difficult environmental conditions as reduced food availability and harsh climatic situations upon reduction of physical activity. Mice that lack GPR50 enter faster torpor and generally display lower body temperatures during sleep and fasting, due to less warmth producing uncoupling protein-1 (UCP1) expression and higher active thyroid hormone levels, which lead to a decrease in body temperature. Furthermore, it has been shown that GPR50 expression can be controlled by leptin levels. These findings support the role of GPR50 in energy homeostasis and propose an additional role of GPR50 in adaptive thermogenesis that might prevent the entry into hypometabolic states.

*Microarray data*

Another way to get information about a protein function are microarray data that try to identify changes in gene expression at the transcriptome level in cells or tissues under specific experimental conditions. A comparative analysis by microarray of mRNA expression in fibroblasts of normal skin and hypertrophic scar could reveal a strong increase of GPR50 expression under hypertrophic conditions in wound healing (Zhang et al, 2010)**.**

A microarray of cells silenced for the regulator of G protein signaling 4 (RGS4) protein, which terminates opioid receptor signaling and is associated with schizophrenia, showed increased expression of GPR50 (Vrajová et al, 2011).

The detection of a highly enriched GPR50 in the trophoblast membrane of placenta proposes a role in pregnancy associated disorders as preeclampsia (Cox et al, 2011).

With regards to cancers, some studies detected an upregulation of GPR50 in early cancer states or tumorigenic tissue in pancreatic neoplasia (Buchholz et al, 2005) and nicotine induced cellular transformation (Bavarva et al, 2013).

#### 2.3.3.3 Molecular function of GPR50

To dissect the molecular role GPR50, proteomic approaches have been used to identify putative binding partners and associated complexes. One example is the yeast two-hybrid (Y2H) screen, which is applicable for the cytosolic part of a protein. In addition, recent techniques were developed that target specifically membrane protein complexes like the TAP Assay (Daulat et al, 2007).

One first hypothesis came up with the emerging evidence that GPCRs form heterodimers (Levoye et al, 2006b). It was discovered, that GPR50 can form heteromers with $MT_1$ and $MT_2$, with a functional influence on $MT_1$ receptors. GPR50 represses melatonin binding to

$MT_1$ and inhibits downstream signaling, which is depending on the GPR50 cytosolic C-terminus, which impairs coupling to G proteins and causes constitutive coupling to β-arrestin. A physiological impact of this heteromer might be found in tanycytes, where colocalisation of GPR50 and the $MT_1$ has been shown. Probably, the seasonally expressed GPR50 (Barrett et al, 2006) can negatively regulate the response to melatonin during the long photoperiod.

This prototypic paper gave rise to the concept that the orphan GPR50 could be an interacting- and activity modulating protein and was supported by further findings in the following years:

With Nogo-A, another interacting protein was revealed in 2009 in a Y2H-screen with the C-tail of GPR50 with both variants in a human adult brain cDNA library (Grünewald et al, 2009). Nogo-A is known to inhibit neurite outgrowth and deviant expression levels were found in schizophrenia, bipolar disorders and Alzheimer disease. Nogo-A and GPR50 are both localized in postsynaptic fractions of neurons and GPR50 overexpression leads to significant increase in neurite outgrowth concerning their number and length and is accompanied by filopodia- and lamellipodia formation. This suggests, that the interaction of GPR50 with Nogo-A might probably block its proper function. Further studies have to establish a connection to brain function and psychiatric disorders.

Another interaction was demonstrated for GPR50 and the transcription factor TIP60 by a Y2H assay of the C-tail with a mouse testis cDNA library (Li et al, 2011). TIP60 functions as a transcriptional co-activator with histonacetylase activity for nuclear receptors as the glucocorticoid receptor. Surprisingly, this interaction occurs in the nucleus, suggesting a nuclear translocation of GPR50. Further studies in this paper proved, that the cytosolic C-terminal part of GPR50 can indeed localize in the nucleus, like its genetic ancestor RNA Pol II. Functionally, it was shown in cellular models and KO mouse, that the presence of GPR50 increases glucocorticoid receptor-dependent gene expression, supporting an involvement of GPR50 in cellular stress response.

The data from the Y2H assay revealed more potential interacting partners implicated in neural developments, stress response, lipid- and steroid metabolism, neurotransmission and signal transduction. Together with other proteomic assays, this can form an ideal basis for future work in order to reveal more molecular functions for GPR50 and to find its place in cellular signaling and protein networks.

# 3. Transforming growth factor β signal transduction

The transforming growth factor β (TGFβ) superfamily of cytokines-induced signal transduction pathway is composed of membrane localized receptors with an intrinsic serine/threonine kinase activity. Activated upon binding of the TGFβ ligand, they phosphorylate their substrates, the Smads, transcription factors, which then translocate to and accumulate in the nucleus, where they regulate the expression of target genes. The actions of TGFβ signaling are essential for maintenance of cellular- and tissue homeostasis, hence supporting its important role in development, growth and differentiation and its pathophysiological implications, for example in cancer development and progression (Feng & Derynck, 2005; Shi & Massagué, 2003b).

## 3.1 The TGFβ signal transduction pathway

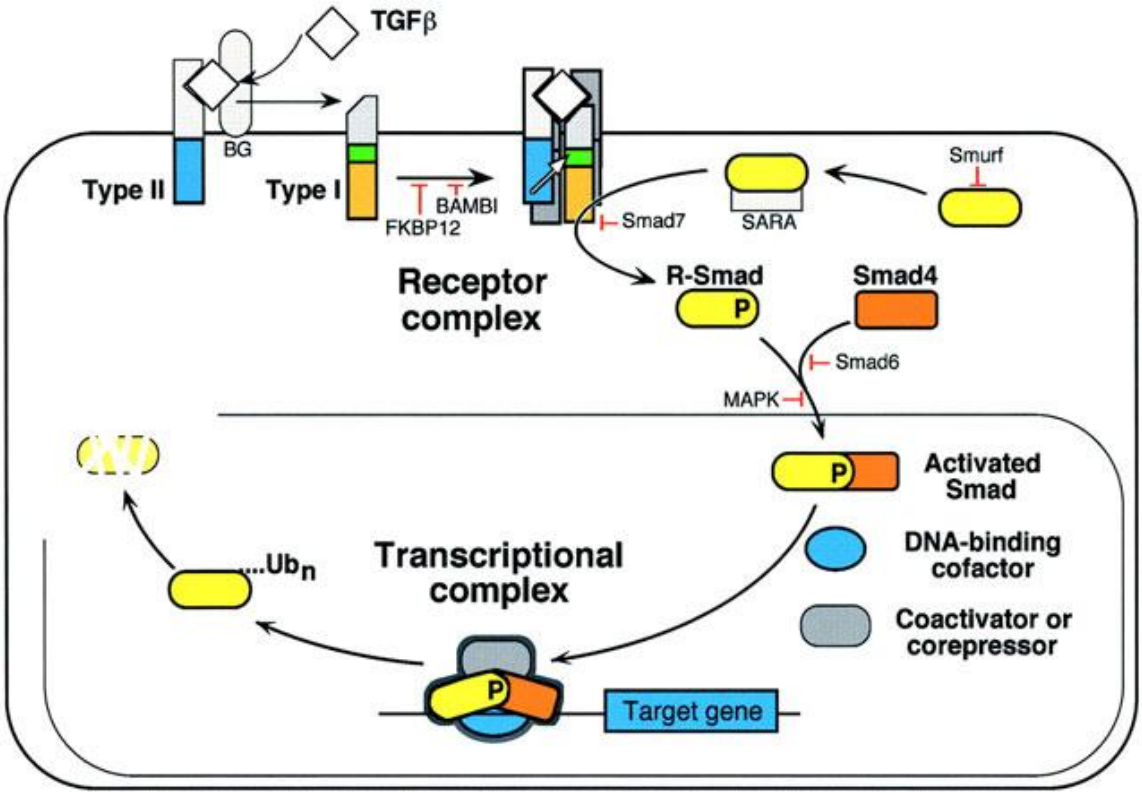



**Figure 14 The TGFβ signaling pathway (Massagué & Wotton, 2000)**

The TGFβ ligand binds to the type II receptor which recruits and transphosphorylates the type I Receptor. Activated type I receptor in turn phosphorylates the Smads which form complexes that translocate into the nucleus and regulate gene transcription. Termination of signaling occurs with proteasomal degradation of the Smads. Proteins that negatively regulate TGFβ signaling (FKBP12, BAMBI, Smad6/7, Smurf, MAPK) are indicated with red bars.

### 3.1.1 TGFβ ligands

In humans, the TGFβ family of cytokines counts 33 members divided into two main categories, discriminated by their sequence similarity and the distinct signaling pathways they activate: (I) TGFβ/Activin/Nodal- and (II) the bone morphogenetic protein (BMP) / growth differentiation factor (GDF) / muellerian-inhibiting substance (MIS) subfamily. The prototypic ones are TGFβ 1, 2 and 3.

Although eliciting various cellular responses, all ligands share common features: they are produced in the cell as dimeric precursors, where they form the C-terminal part of a propeptide. Though getting cleaved during secretion, they get released into the extracellular matrix (ECM) still associated to the N-terminal part (therefore also named the latency-associated polypeptide, LAP) and further proteins like the latent-TGFβ-binding-protein (LTBP), retaining the TGFβ from binding to their receptors. The activation process requires either acidic conditions, cleavage by extracellular proteases (as metalloproteases) or interaction with the Arg-Gly-Asp (RGD) sequence of integrins (Annes et al, 2003). The active TGFβ ligands form dimers and one monomer is composed of several β strands that are associated by disulfide-bonds, a structure known as the cysteine-knot (Sun & Davies, 1995).

The presence of the TGFβ ligand as a dimer suggests that the ligand is binding to homodimeric structures of its cognate TGFβ receptors.

### 3.1.2 TGFβ receptors

The receptors for the TGFβ ligands are membrane-localized kinases with serine/threonine activity, thus forming the only existing family of receptor serine/threonine kinases (RSTK). In humans, 12 genes are encoding for the different receptors, divided into two subfamilies, with seven type I and five type II receptors (Manning et al, 2002). The different type I receptors are further subdivided upon the ligand-specific downstream cascades, they activate. The small number of receptors compared to the high number of ligands is quite surprising, but the existing specificity for each of the 33 ligands is established by the receptor promiscuity, allowing different heteromeric combinations, as illustrated in Figure 15. The best-described and -studied system is the one for the TGFβ-1/2/3 ligands that bind TβRII and signal through TβRI (former ALK5).

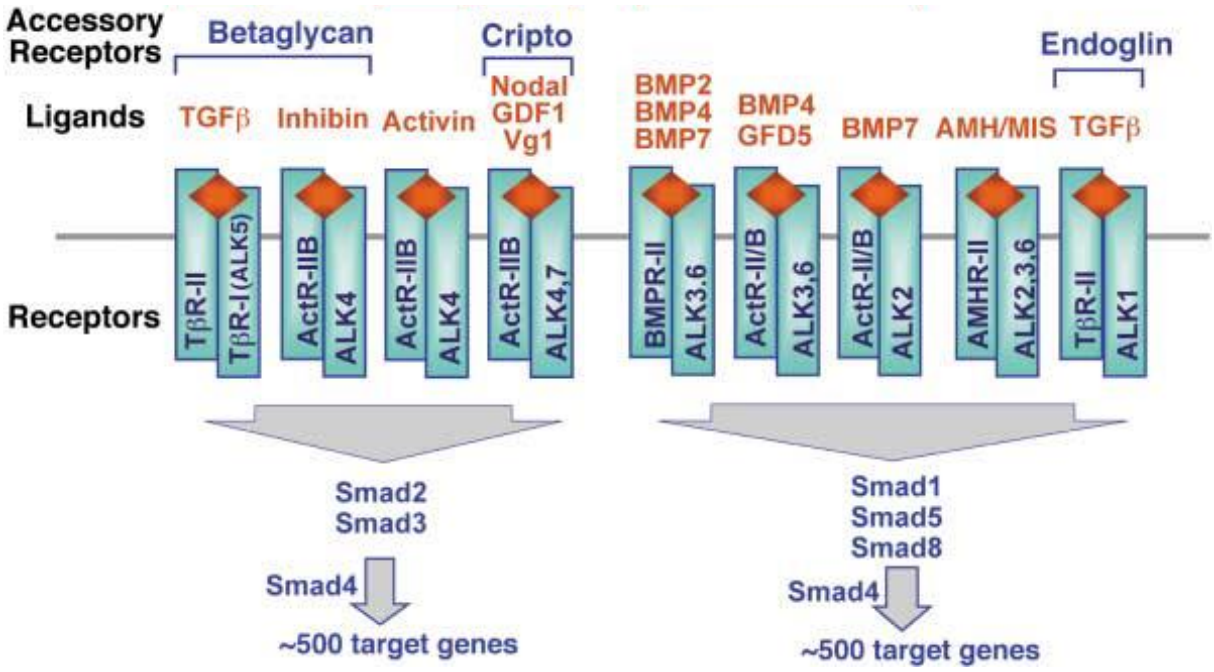



**Figure 15 TGFβ ligands and their receptors (Shi & Massagué, 2003b)**

The TGFβ superfamily of cytokines is composed of 33 members, divided into two groups, the TGF/Activin/Nodal and the BMP/GDF/MIS group. These ligands are bound by only 12 receptors (seven RI and five RII), which are either activating TGFβ/Activin/Nodal- or BMP/GDF/MIS-dependent signaling. Different Type II receptors can combine with different RI receptors, thus forming combinations that are specific for each ligand. Furthermore, accessory receptors like Betaglycan, Cripto and Endoglin are implicated in ligand activation.

*Receptor structure*

The TGFβ receptors TβRI and TβRII receptors are composed of 503 (TβRI) or 567 (TβRII) amino acids, respectively. They are divided into a short, cysteine-rich, extracellular domain responsible for ligand binding, a single-pass transmembrane part and a long dominating cytosolic portion which is enzymatically active (Massague, 1998). Interestingly, sequence analysis and comparison revealed that the receptors have a dual kinase specificity, with a stronger affinity to phosphorylate Ser- and Thr-residues, but a phosphorylation on Tyr-residues has also been demonstrated for the RII. (Hanks & Hunter, 1995; Lawler et al, 1997). Between the TβRI and TβRII exist important structural differences, which are reflected in the mechanism of activation: the TβRI has a characteristic, conserved glycine and serine rich GS region preceding the kinase domain, which is composed of several serines and threonines that form the target of phosphorylation by the TβRII. This GS domain is the activity-regulating unit and the flexible ankle in conformation, explaining the constitutive activity of the TβRII

and the necessity of TβRI being activated. The TβRI also carries an additional L45 loop for Smad recognition (Figure 16).

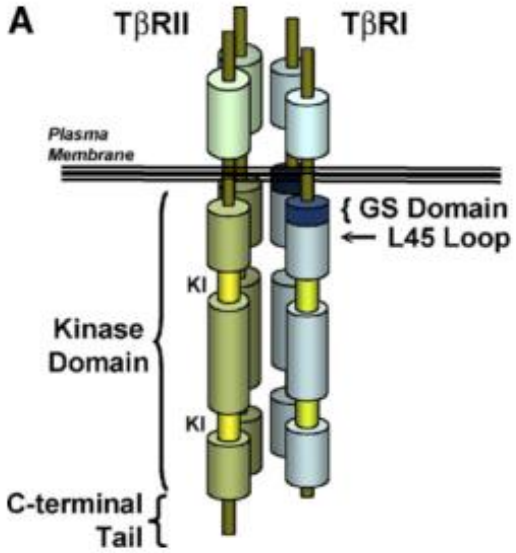



**Figure 16 Schematic overview of the TβRI- and TβRII structure (Runyan et al, 2006)**

The TGFβ receptors are composed of an extracellular part, a single transmembrane spanning segment and a long cytosolic tail. The cytosolic part bears the kinase domain. In TβRI, additional characteristic parts are the GS domain which is the phosphorylation target for TβRII and the L45 loop for Smad binding.

*Mechanism of ligand binding - 2 different models*

The different type I- and type II- receptors also diverge in their mode of ligand binding and activation, depending on the ligand. The BMP ligand family has a quite weak affinity for both of their receptors, resulting in a cooperative binding model, where the ligand binds both receptors together to form a stable complex (Rosenzweig et al, 1995). In contrast, the loss of the Phe85 residue, which is creating hydrophobic interactions between ligand and receptor in the BMP receptors (Kirsch et al, 2000), the TGFβ receptor TβRI has no ability to bind the TGFβ ligand alone. TGFβ has a much higher affinity for binding to the TβRII (Attisano et al, 1993). Thus, complex formation occurs in a sequential manner, induced by a strong binding of TGFβ ligand to the TβRII, which subsequently enables the recruitment of the TβRI to the complex (Hart et al, 2002).

*Mechanism of receptor activation*

The heteromeric complex composed of ligand, homomeric TβRII and homomeric TβRI, which is formed after ligand binding, induces a juxtaposition that favors the

transphosphorylation of the inactive TβRI by the constitutive active TβRII (Franzen et al, 1993; ten Dijke et al, 1994). These phosphorylations occur in the characteristic GS domain of TβRI (Wieser et al, 1995), rendering the TβRI in the active conformation and enabling the signal propagation through its kinase function. This ligand binding induced phosphorylation of TβRI by TβRII is absolutely required for signal transduction (Luo & Lodish, 1996; Okadome et al, 1994; Vivien et al, 1995). In contrast to the TβRII, no evidence for an *in vivo* autophosphorylation of TβRI exists (Weis-Garcia & Massague, 1996; Wieser et al, 1995; Wrana et al, 1994). This phosphorylation of TβRI in its GS domain induces a conformational inhibition-to-activation switch, a crucial step in transmission of TGFβ signals (Huse et al., 2001a). The phosphorylations lead to a conformational change in the GS domain, accompanied by a disappearence of the binding site of the negative regulator FKBP12 (see Chapter 3.2.3.4.), which causes its dissociation. Consequently, the nine amino acid L45 loop, which forms the binding surface for the TGFβ effectors, the Smads (Persson et al, 1998), becomes accessible.

### 3.1.3 Smad transcription factors

The signal of TGFβ is then transmitted via the type I receptors to their substrates, the Smads (short for: small mothers against decapentaplegic homolog), which first have been discovered in 1996 (Derynck et al, 1996). The family of Smad Proteins is composed of eight different members, divided into three different classes: (I) the receptor-regulated R-Smads, Smad1, 2, 3, 5 and 8 which bind to the type I receptor, are a substrate for phosphorylation and form homo- or heteromeric complexes. The R-Smads are furthermore categorized upon their ligand response: Smad2 and Smad3 are phosphorylated after TGFβ ligand stimulation, while Smad1, 5 and 8 are BMP-ligand effectors; (II) the common-mediator co-Smad, Smad4, which is indispensable for formation of nuclear translocating complexes with the R-Smads that together bind to DNA and influence gene transcription. (III) The inhibitory I-Smads, Smad6 and 7, compete with R-Smads on receptor binding and target the receptor complex for degradation by recruiting ubiquitin ligases. Smad7 can bind all receptor I subtypes, whereas Smad6 has a specificity for the BMP binding receptor I (Heldin & Moustakas, 2012).

*Structure and sequence of the Smads*

The R- and Co-Smads comprise about 500 to 600 amino acids and are divided into two major structural entities, the N-terminal MH1 (for Mad-homology) and the C-terminal MH2

domain, connected through a less conserved linker domain (see Figure 17). The MH1 domain of both, R-Smads and Co-Smads, allows nuclear translocation and mediates DNA binding. The MH2 domains of all Smad classes are similar in their sequence and are, in the case of R- and I-Smads, responsible for receptor binding, complex formation with other Smads, nuclear shuttling and transcriptional activation. Also most of the protein interactions are maintained by the MH2 domain. A distinct feature of the R-Smads is their C-terminal SXS motif, which forms the target for phosphorylation by type I receptors. This strong homology of the different MH2 domains also allows the I-Smads to bind the receptor. But lacking the SXS motif explains a part of their inhibiting effect: in the absence of this phosphorylation, no downstream signaling is possible. The domains are connected by the less conserved proline-rich linker region, which is important for crosstalk with other signaling pathways by being a phosphorylation target and implicated in interaction with other proteins (Moustakas & Heldin, 2009).

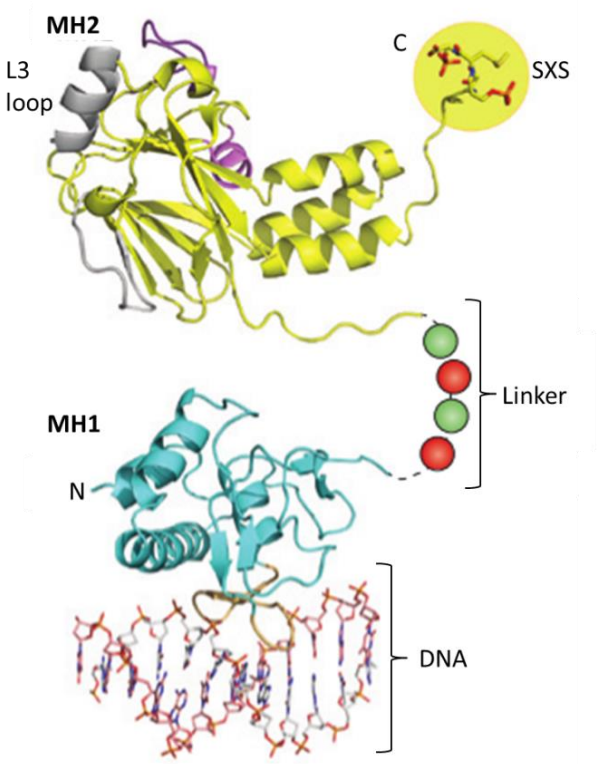



**Figure 17 Smad3 structure (Massague, 2012; Shi & Massagué, 2003b)**

The Smads are composed of two main domains, MH1 and MH2 that are connected by a flexible linker region. The MH1 mediates DNA binding and the L3 loop in the MH2 domain is responsible for RI binding in R- and Co-Smads. The C-terminal SXS motif target in R-Smads is the target of phosphorylation by RI (indicated by yellow circle). The linker region is a substrate for phosphorylation by other kinases.

*Receptor recognition, activation and nuclear translocation of the Smads*

The type I receptor-Smad recognition is obtained between the exposed L45 loop of the type I receptor and the L3 loop in the MH2 domain of the Smads. The interactions between the L45 and the L3 loop and their specific sequences in each type I receptor and Smad-subtype explain the preference of Smad 1,5 and 8 to bind to BMP receptors and of Smad2 and 3 to bind TGFβ receptors (Feng & Derynck, 1997; Wu, 2000). After binding to the type I receptor, the Smads get directly phosphorylated by the type I receptor in the serines of the C-terminal SXS motif (Abdollah et al, 1997; Kretzschmar et al, 1997; Macias-Silva et al, 1996), which is enabling the complex formation with Smad4 and leading to the exposure of a domain that is important for nuclear translocation (Wu et al, 2001; Xu et al, 2000).

After phosphorylation has triggered the formation of heteromeric complexes (mainly composed of two R-Smads and one co-Smad4), they translocate to the nucleus. This nuclear import is mediated by a conserved nuclear localization signal (NLS) of the R-Smads (Xiao et al, 2000a), leading to translocation either via the uncommon Importin β pathway for Smad3 (Xiao et al, 2000b), or, in the case of both Smad2/3, by direct binding to the nucleoporin-complex proteins (Xu et al, 2002). For Smad4, the classical importin α pathway is supposed. An important feature is the occurrence of dynamic nuclear-cytosolic shuttling for the co- and R-Smads, permitting repeated cycles of receptor-binding, phosphorylation, complex formation and translocation, explaining the persistence of the TGFβ signal for several hours (Inman et al, 2002; Pierreux et al, 2000).

### 3.1.4 TGFβ-dependent regulation of gene expression

Once located in the nucleus, Smad proteins mediate the TGFβ ligand-dependent regulation of gene expression by acting as transcription factors.

*DNA binding*

Binding of Smads to DNA occurs for all R-Smads (except Smad2) and the co-Smad through a common DNA harbored binding motif: the Smad-binding-element (SBE) that is consisting of only 5 bases, 5'-CAGAC-3' (Yingling et al, 1997). This interaction is carried out by a conserved β-hairpin structure in the MH1 domain. In contrast, Smad2 is bearing an additional 30 amino acid long insert, abolishing its DNA binding ability and explaining the requirement of additional proteins for DNA binding, like the forkhead transcription factor FoxH1. Together they bind DNA at the Smad2-activin-response-element (ARE) together with the

activin-response factor ARF (Chen et al, 1996). With regards to this quite short and unspecific sequence and the weak intrinsic DNA binding affinity of Smads, further mechanisms are essential to achieve specificity in their regulation of transcription.

*Regulation of activation and repression*

This regulation is made up by the interaction with transcriptional co-factors, who can bind DNA and other transcription factors at once. The interaction with these co-activators or co-repressors occurs between the Smad-interacting-domain (SID) in the MH2 domain and a proline-rich Smad interacting motif (SIM), found in some transcription factors, as FoxH1. The interactions with co-factors are responsible for establishing the different Smad responses in a cell type- and context-dependent manner. These interacting proteins can be either (I) general transcription factors, that regulate activation or repression and amplitude, like the co-activators as CBP/p300 and co-repressors like the TGFβ-induced factor TGIF or c-Ski and SnoN that can also modulate chromatin structure through their intrinsic histoneacetylase- or histonedeacetylase-activity respectively, or (II) specific transcription factors, that allow the regulation of specific genes, like proteins of the basic-helix-loop-helix bHLH family like TFE3, the basic leucine zipper domain bZIP family like ATF3, c-Fos, c-Jun and c/EBP, the forkhead family, homeodomain proteins like Dlx and Hox, nuclear receptors like the estrogen receptor, zinc finger proteins like GATA and Sp1, or signal integrators, which are responsible for mediating crosstalk with other signaling pathways as β-catenin, HIF, NFκB, p53 and SRF (Feng & Derynck, 2005).

*Regulated genes*

These various interactions and the sequence alterations in the different R-Smads enable for each of it a regulation of an own subset of genes, which underlines that the Smads, dependent on the cellular context, can control the expression of a myriad of genes. Some of the abundant events are activation of the cell cycle inhibitors p21kip and p15Ink4b or the extracellular matrix protein plasminogen activator inhibitor PAI-1 and the repression of c-myc. Furthermore, some of the regulated genes are “selfenabling genes” which later get involved in regulating or participating in the TGFβ signaling pathway, like Smad6/7 or ATF3. An overview is given in Table 2.

**Table 2 TGFβ regulated genes in epithelial cells (Siegel & Massague, 2003)**

Overview of common regulated genes in epithelial cells of breast, skin and lung

| Functional group | TGF-β response | Genes |
|---|---|---|
| Cytostatic programme | Upregulated<br>Repressed | *CDKN2B*, *CDKN2A*<br>c-*MYC*, *ID1*, *ID2*, *ID3* |
| Extracellular matrix | Upregulated | *PAI1*, *uPA*, *Col VI-A1*, *ADAM19*, *ITGα5*, *ITGβ6* |
| Paracrine network | Upregulated<br>Repressed | *IL11*, *VEGF*, *CTGF*, *JAG1*, *FSTL3*, *ANGPT4*<br>*IL1β*, *BMP4* |
| Signalling network | Upregulated<br>Repressed | *BMPRII*, *VDR*, *EPHB2*, *RHOGEF114*, *MEKK4*<br>*LDLR*, *PGE-R4*, *βAR-2* |
| Transcriptional network | Upregulated<br>Repressed | *ETS2*, c-*JUN*, *JUNB*, *ATF3*, *GADD45B*, *PIM1*, *MAD2*, *MAD4*<br>*C/EBPδ*, *MRG1*, *TRIP-Br2* |
| Other responses | Upregulated<br>Repressed | *TBX3*, *MN1*, *IGl*, *SIAT4A*<br>*SPRY2*, *IAP3* |
| Negative feedback | Upregulated | *SMURF1*, *SMURF2*, *SMAD7*, *SNON* |

*Table adapted from REF. 11. The gene responses are subdivided into functional groups and those that are upregulated or downregulated by TGF-β are indicated. Only a few gene responses are specifically devoted to mediating the cytostatic response of these cells. Other gene responses regulate the interaction of the target cell with the environment, either by controlling the production of cytokines and extracellular-matrix components, or by modifying the signalling networks and transcriptional regulators that sense and process external signals including the TGF-β signal itself. *ADAM19*, a disintegrin and metalloproteinase domain 19; *ANGPT4*, angiopoietin-like 4; *ATF3*, activating transcription factor 3; *βAR*-2, adrenergic β2 receptor; *BMP4*, bone morphogenetic protein 4; *BMPRII*, bone morphogenetic protein receptor type II; *C/EBPδ*, CCAAT/enhancer binding protein (C/EBP) δ; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; *Col VI*-A1, collagen, type VI, α1; *CTGF*, connective-tissue growth factor; *FSTL3*, follistatin-like 3; *GADD45B*, growth arrest and DNA-damage-inducible β; *IAP3*, apoptosis inhibitor 3; *ID*, inhibitor of DNA binding; *IGλ*, immunoglobulin λ; *IL*, interleukin; *ITGα5*, integrin α5; *ITGβ6*, integrin β6; *JAG1*, jagged 1; *JUNB*, JUN B proto-oncogene; *LDLR*, low-density-lipoprotein receptor; *MAD*, MAX dimerization protein; *MEKK4*, mitogen-activated protein kinase kinase kinase 4; *MN1*, meningioma (disrupted in balanced translocation) 1; *MRG1*, myeloid ecotropic viral-integration-site-related gene 1; *PAI1*, plasminogen activator inhibitor type 1; *PGE-R4*, prostaglandin E receptor 4; *RHOGEF114*, RHO-specific guanine nucleotide exchange factor p114; *SIAT4A*, sialyltransferase 4A; *SMURF*, SMAD ubiquitylation regulatory factor 1; *SPRY2*, sprouty homolog 2; *TRIP-Br2*, transcriptional regulator interacting with the PHS-bromodomain 2; *TBX3*, T-box 3; *uPA*, urokinase-type plasminogen activator; *VDF*, vitamin D receptor; *VEGF*, vascular endothelial growth factor.

Besides the regulation of genes that encode for proteins, Smads were also reported, to be involved in the regulation of the expression of certain microRNAs or other regulatory nucleic acids (Blahna & Hata, 2012).

*End of signaling*

Once the Smads exerted their role as signal transducers by regulating gene expression, the question arises, how signaling gets terminated. With regards to Smads, either dephosphorylation that induces nuclear export or their degradation after ubiquitination leads to abolishment of signaling. Other mechanisms involve the action of interacting proteins or negative regulators are discussed in chapter 3.2.4

## 3.2 Regulation of TGFβ signaling - the establishment of signaling specificity and diversity

The TGFβ signaling pathway is, in addition to being the only described receptor serine/threonine kinase pathway, also marked by other characteristic features, discriminating

it from other classical signal transduction pathways. In the TGFβ pathway, we observe a straight forward linear signaling flow after ligand binding from type II receptor to type I receptor via the Smads into the nucleus, while other pathways like the RTK EGF-Ras-ERK pathway, display more complex cascades with a multistep transmission including several enzymes and scaffolding proteins. This case is termed "wiring" with a non-linear signal amplification as a consequence (Schmierer & Hill, 2007). However, in contrast to RTK pathways, the TGFβ signals are converted into a cellular effect in a slow manner, grounded in the need to establish a Smad nuclear accumulation for a response to the ligand. But signals are highly sustained, because signal termination necessitates not only a switch-off mechanism, but a longtime degradation process. Comparing these distinct modes of signal transduction pathways gives rise to the question, how specificity and fine-tuning of signaling are obtained in the case of TGFβ signaling. Furthermore, the contrast of the large number of ligands, their cognate receptor combinations and the small number of signal transducing Smads stirs up the issue how they can be capable of reflecting this ligand diversity. The solution lies in three characteristic features of the TGFβ signaling pathway: (I) the pleiotropy of interaction partners and regulating proteins (II) their coordination and (III) the context dependency based on the cell type specific distribution of signaling components, which place the TGFβ signaling from a linear pathway into a network (Massague, 2012). In the following, these different components of the network and their TGFβ-signaling specific effects will be explained:

### 3.2.1 Mechanisms of regulation

The regulation of the TGFβ signaling can be obtained through a multitude of regulatory mechanisms:

*(I) Regulation of expression of pathway components*

The amount of available signaling components is a critical part of signaling, enforcing a regulation on the genetic level. This regulation of expression of their genes is often induced as an effect of other signaling pathways. For example, the expression of inhibitory Smads is regulated through cytokines via the JAK/STAT (Ulloa et al, 1999) or the tumor necrosis factor (TNF)-α/ nuclear factor (NF) κB pathway (Bitzer et al, 2000). Moreover, epigenetic regulatory mechanisms gain more attention in influencing the amount of translated protein for example through miRNA regulated gene expression.

*(II) Enzymatic covalent modification of pathway components*

Enzymatic actions are a much quicker possibility to regulate the activity of a signal transduction pathway than genetic events. Numerous possibilities of modifications like phosphorylation/dephosphorylation, ubiquitylation/deubiquitylation/sumoylation, acetylation, methylation, glycosylation and ADP-ribosylation of the TGFβ signaling pathway components have been reported (Xu et al, 2012). Especially the dynamics and the interplay of these posttranslational modifications add a strong touch of flexibility to TGFβ signaling.

*(III) Protein interactions*

In addition to these protein-mediated modifications, direct interactions of the TGFβ signaling core proteins with other proteins can be another possibility for affecting their activity or subcellular localization.

*(IV) Crosstalk*

A further step is the integration of the TGFβ signaling pathway into the signaling network of a cell, meaning the crosstalk with other signaling pathways, which will be discussed in chapter 3.3.

These modes of regulation can occur on every level of TGFβ signaling, which is going to be dissected in the following:

### 3.2.2 Ligand activity and availability

The TGFβ ligand, existing as latent complex associated with the LAP and anchored by LTBPs in the extracellular matrix (see 3.1.1), requires further processing for activation. This occurs either by cleavage through extracellular proteases like metalloproteases or upon integrin interaction - steps which can be subject of regulations *(ten Dijke & Arthur, 2007).* Furthermore the interaction of the ligand with other extracellular or transmembrane proteins can interfere with ligand activation: (I) Soluble proteins like noggin (acting on BMP7) or inhibin (acting on activin) function as ligand traps, that sequester the ligand and thus impede receptor binding (Groppe et al, 2002), (II) membrane bound proteins that often operate as co-receptors (see also Figure 15): one group of them is often referred as type III receptor, receptors with ligand binding ability but without intrinsic signaling activity, like betaglycan and endoglin. Betaglycan, a membrane anchored proteoglycan (Brown et al, 1999a) can either participate in presenting the TGFβ ligand for the receptor (Lopez-Casillas et al, 1993) or otherwise, after its shedding into the extracellular matrix, apply antagonistic affects as a

ligand scavenger (Lopez-Casillas et al, 1994). The homologic endoglin is mainly expressed in endothelial cells (Cheifetz et al, 1992), Cripto proteins binds the Nodal and Activin ligands (Gray et al, 2003). Connective-tissue growth factor (CTGF) is a selfenabling gene induced by TGFβ, that enhances its receptor binding, with an important role in ECM formation (Abreu et al, 2002). Another ligand availability regulating protein is the pseudoreceptor Bambi (Onichtchouk et al, 1999), which acts as a decoy receptor for BMP and competes with the BMP-RI upon incorporation into the heteromeric receptor complex.

### 3.2.3 Regulation of TGFβ receptor activity

A huge number of proteins is implicated into the modulation of receptor activity, either by exerting enzymatic modifications or regulation of activity and downstream signaling due to a direct interaction.

#### 3.2.3.1 Enzymatic receptor modifications

*Regulation by ectodomain shedding*

Enzymatic shedding can have, dependent on the involved enzymes, different possible outcomes. Shedding of the TβRI via the metalloproteinase TACE/ADAM17 leads to a decrease of cell surface receptor levels and thus negatively influences the sensitivity of cells for the ligand (Liu et al, 2009a).

*Regulation by phosphorylation and dephosphorylation*

In addition to the transphosphorylation of type I receptors by type II receptors, which is crucial for signal transduction, further regulatory phosphorylations on both receptors have been described. The TβRII can autophosphorylate itself not only in an activity-promoting- (on Ser213 and 409), but also in an inhibiting manner (Ser416). Also tyrosine phosphorylation can be found, either as a result of the dual specificity by an autophosphorylation that is supposed to be necessary for activation (Lawler et al, 1997), or it can be carried out by other proteins like Src what leads so subsequent activation of the p38 protein (Galliher & Schiemann, 2007). For TβRI a phosphorylation on Ser165 was reported, that is associated with modulation of the cellular response (Souchelnytskyi et al, 1996). These regulatory phosphorylations in both receptors are often involved in the creation of binding sites for other interacting partners.

The other way round, desphosphorylation is a mean to inactivate the receptor and to arrest signaling. Concerning this, evidence exists that proteinphosphatases PP1c, together with Smad7 binding (Shi et al, 2004), and PP2a are involved enzymes (Griswold-Prenner et al, 1998).

*Regulation by ubiquitylation and sumoylation*

Typically, polyubiquitylation targets proteins for proteasomal degradation by the sequential action of the enzymes E1 and E2 and the ubiquitin ligase E3. For proteasomal degradation of the TGFβ receptor complex, the inhibitory Smad7 functions as an adaptor by recruiting Smurf1/2 (Kavsak et al, 2000) and WWP1 (Komuro et al, 2004) ubiquitin ligases. Reversion of this effect can be achieved by deubiquitinating enzymes, as the deubiquitinases USP15 (Eichhorn et al, 2012) and UCH37 (Wicks et al, 2005). In addition to the polyubiquitylation, evidence exist for a regulatory receptor monoubiquitylation: it was shown that the combination of metalloproteinase ADAM12/TACE-dependent TβRI shedding and a TRAF6 E3-ligase-mediated monoubiquitylation leads to a nuclear translocation of the TβRI, where it regulates gene transcription (Mu et al, 2011). Another covalent protein modification is sumoylation that influences subcellular localization. Sumoylation by Ubc9 was found on phosphorylated TβRI that further enhanced its activity (Kang et al, 2008).

#### 3.2.3.2 Regulation of subcellular localization of the receptors

Another important step in signaling is the subcellular localization of receptors. Compared to RTK signaling, an increase of internalization and the constitutive formation of signaling endosomes as a consequence of ligand binding is not described for the TGFβ signaling pathway. Nevertheless, receptor endocytosis exists to modify activity for which two distinct mechanism were shown (Di Guglielmo et al., 2003): (I) clathrin-dependent internalization, involving a dileucin motif which locates the TβRI to early-endosome-antigen1 (EEA) positive endosomes that can cycle back to the membrane via Rab11 endosomes. This mode of internalization is a way of receptor recycling and can favor enhancement or endurance of signaling. In contrast, (II) clathrin-independent internalization via calveolar-positive vesicles in lipid rafts is favoring proteasomal or lysosomal degradation of the receptor complex. An additional mechanism for degradation of the receptor complex is the possibility of a β-arrestin 2-mediated internalization, a protein responsible for GPCR trafficking (see Chapter 1.3.), carried out by interaction of the short cytoplasmic tail of the TβRIII betaglycan with β-arrestin 2 (Chen et al, 2003).

#### 3.2.3.3 Regulation by interacting proteins

During the last years a huge number of proteins that interfere with TGFβ signaling by interaction with receptors have been identified. Their effects can be enhancing and repressing and their modes of action and interaction are various: they can act as scaffolds, influence subcellular localization, others direct signaling towards non-canonical pathways or mediate the crosstalk with other pathways (Runyan et al, 2006).

(I) NEGATIVE REGULATION

Interactions with proteins exerting a negative regulation can either act to prevent the occurrence of signaling or to restrict its duration.

**Bambi**, is the BMP decoy receptor, which inhibits downstream signaling by competing with BMP-RI for binding to BMP-RII as a result of their sequence homology. This leads to the formation of inactive Bambi/RII complexes (Onichtchouk et al, 1999). Later findings suggest cooperative mechanism of Smad7 and Bambi in inhibiting signaling what might extend the role of Bambi towards TGFβ-ligand-induced signaling (Yan et al., 2009).

**Smad 6 and 7**, the inhibitory Smads are signaling antagonists which are expressed under the control of R-Smads, thus being part of a negative feedback loop of TGFβ signaling (Hayashi et al, 1997; Nakao et al, 1997a). Like the R-Smads, they bind to the receptor (Hanyu et al, 2001), but lacking the SXS motif, they cannot be phosphorylated, what is resulting in a competition with R-Smads, that blocks downstream signaling (Hayashi et al., 1997). Furthermore, they can serve as scaffold for the recruitment of other proteins: GADD34 which binds the phosphatase PP1c and causes receptor dephosphorylation (Shi et al, 2004), the E3 ligases Smurf1 and 2 (Kavsak et al, 2000; Suzuki et al, 2002) and WWP1 (Komuro et al, 2004), that are polyubiquitylating the receptors, which triggers the proteasomal degradation of the complex. Other synergizing proteins are STRAP, which binds Smad7 and can also bind TβRI and TβRII with its WD40-domain repeats and negatively regulates Smad2/3 (Datta et al, 1998) and YAP65, a member of the Hippo pathway, that increases the TβRI-Smad7 association (Ferrigno et al, 2002). Further roles are also described for inhibiting complex formation of R-Smads and DNA (Zhang et al, 2007).

**DRAK2 (**DAP kinase-related apoptosis-inducing protein kinase) is a recently identified TGFβ-inducible protein kinase, which constitutively interacts with TβRI and gets increased

upon ligand stimulation. DRAK2 seems to be an important player in attenuation of TGFβ signaling (Yang et al, 2012).

Further proteins that were described to negatively regulate signaling upon receptor interaction are the chimeric oncoprotein ETV6-NTRK3 (Jin et al, 2005) and the RTK TrkC (Jin et al, 2007) that both interact with TβRII, thus preventing interaction with TβRI. Dpr2 binds to the receptor I and targets it for lysosomal degradation (Su et al, 2007b), and c-Ski, that beside its nuclear function (see below) can also negatively regulate the receptors (Ferrand et al, 2010). Further examples are described in Table 2.

#### 3.2.3.4 FKBP12 as a signaling-preventing negative regulator

A regulator, which holds an important role as inhibitor of basal TGFβ signaling activity is the immunophilin FKBP12. The immunophilins are a group of proteins that can bind immunosuppressive substances and function as peptidyl-prolyl-cis-trans-isomerases (PPI). Their main role is associated with protein folding and -refolding and stabilization of distinct protein conformations. FKBP12 (FK506 binding protein 12) is able to bind the macrolides FK506 (tacrolimus) and rapamycin and has a size of 12kDa. The structure of FKBP12 is highly conserved among the different species and is composed of five β turns and two extremities, the 40s and 80s loop, flexible regions and essential parts for binding to other proteins. By being a PPI, the recognition sequence in other proteins is composed of at least one proline, but preferentially composed of a Pro-Leu dipeptide. Most functions of FKBP12 arise from its complex forming properties together with FK506 and calcineurin, a calcium activated phosphatase, or together with rapamycin FKBP12 can bind PI3K like kinases RAFT. Another function is the regulation of $Ca^{2+}$ channels by binding to ryanodine- and IP3-receptor-calcium channels and influencing their closure kinetics (Ivery, 2000).

*The interaction of FKBP12 with the TβRI*

An interaction that is maintained independently from macrolides is the one with the TβRI. By performing yeast two-hybrid assays all five type I receptor family members were found to interact with FKBP12, but none of the type II receptors (Wang et al, 1994). The interaction has been further studied especially for the TβRI. FK506 and rapamycin inhibit this interaction, suggesting a competition for the same binding site in FKBP12. Mutation studies have shown, that FKBP12 binds to the $^{193}$Leu-Pro-Leu-Leu$^{196}$ motif in the GS domain of the

TβRI, what is coherent with its PPIase-activity, requiring a Leu-Pro motif for interaction (Charng et al, 1996; Chen et al, 1997). This got support by the publication of the crystal structure (see Figure 18), revealing more details about the interaction (Huse et al, 1999): The extreme 40s and 80s loop in FKBP12 recognize a distinct structure, the VI β turn of the TβRI, lying the GS domain and bearing the $^{193}$LPLL$^{196}$ motif as recognition site for FKBP12. FKBP12 is directly interacting with two Leu residues (195 and 196) and Pro194 is assisting in stabilization. Additionally, a contact is made between the exposed His87 and Pro88 of the 80s loop in FKBP12, which directly contact the β3 and β4 sheet in the L45 loop, the surface for R-Smad binding. Upon this interaction, TβRI gets stabilized in an inactive conformation by the formation of an inhibitory wedge in the GS domain, providing a structural explanation for FKBP12 locking the inactive conformation and constraining the TβRII-mediated transphosphorylation in the GS domain and R-Smad binding to the L45 loop.

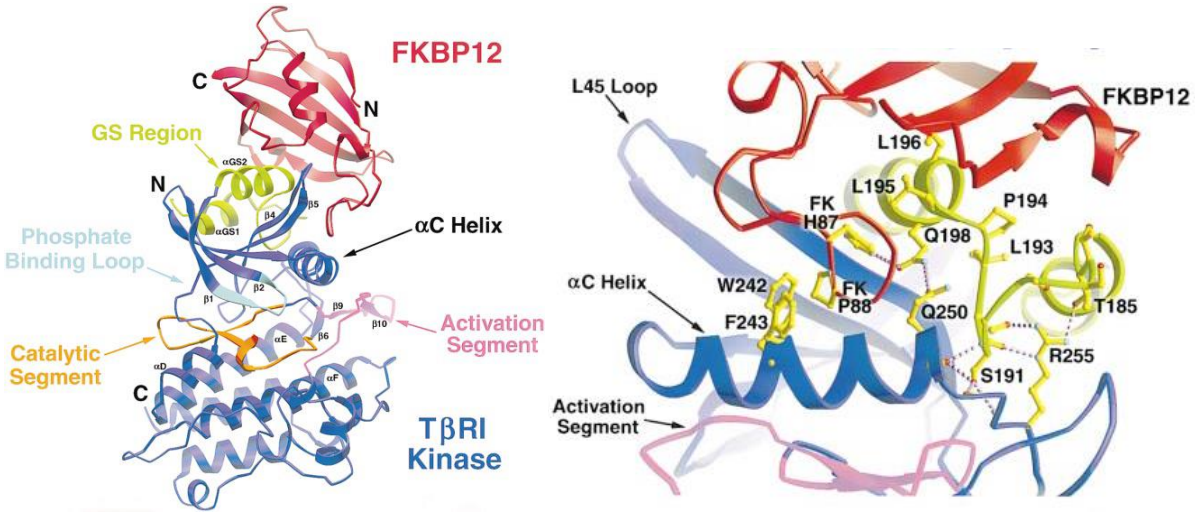



**Figure 18 Crystal structure of FKBP12 and TβRI (Huse et al, 1999)**

The crystal structure of FKBP12 and the cytoplasmic domain of the TβRI provides important information about the interacting domains. FKBP12 (in red) contacts the TβRI with its 40s and 80s loop in the GS domain of the TβRI at the $^{193}$LPLL$^{196}$ sequence (light green). Another side of interaction is made between the H87 and P88 of the 80s loop in FKBP12 and the L45 loop of the β turns 3 and 4 (in blue).

*Functional effect of the TβRI-FKBP12 interaction*

If the binding site for FKBP12 in the TβRI is mutated, a basal, ligand-independent signaling can be observed. That is suggesting a role of FKBP12 in preventing signaling instead of actively participating in the ligand-induced response (Charng et al., 1996). Treatment with FK506 also leads to tonic TGFβ signal transduction and an increased response to low doses of

ligand, whereas overexpression of FKBP12 can block or diminish the TGFβ signaling response, an effect also described for BMP receptors (Gruendler et al, 2001; Spiekerkoetter et al, 2013). It was show that depletion of FKBP12 from the TβRI induces its hypersensitivity for TβRII-mediated transphosphorylation in the GS domain (Chen et al, 1997). These findings suggest that FKBP12 is important for blocking basal TGFβ signaling in the absence of ligand. FKBP12 KO mice further support this function of FKBP12: These mice display defects in heart function and neural tube closure (Shou et al., 1998) and mouse-derived cells display a higher amount of TGFβ downstream signaling effects such as increased p38 phosphorylation (see Chapter 3.3.1) and p21 expression, leading to increased cell cycle arrest (Aghdasi et al, 2001; Shou et al, 1998).
Additional functions of the TβRI-FKBP12 interaction are the blocking of TβRI internalization (Yao et al, 2000), probably through an orchestration of TβRI degradation in complex with Smad7 (Yamaguchi et al, 2006) or still unsolved issues as the influence of its PPI-activity or a role in the recruitment of other proteins.

*Mechanism of FKBP12 release*

A still unsolved part of the puzzle is the exact mechanism that triggers FKBP12 release. Studies have shown that the release of FKBP12 occurs (I) upon ligand stimulation and (II) by transphosphorylation of the GS domain in TβRI by TβRII, but the exact point of FKBP12 release could not be determined. This release should be preceding any GS domain phosphorylation event, because mutating all GS domain phosphorylation sites does not prevent the release of FKBP12. But a deletion of the TβRII-kinase domain locks FKBP12 bound to the TβRI, evoking the possibility of other TβRII-mediated events like phosphorylation of another residue outside the GS domain or the binding of other proteins, that trigger the release of FKBP12 (Wang & Donahoe, 2004; Wang et al, 1996).

(II) POSITIVE REGULATION

Another group of interacting proteins are positive regulators that are either essential for TGFβ signaling or that can potentiate and prolong signaling.

**TRAP1 (TGFβ receptor associated protein) and TLP (TRAP1 like protein)** are two proteins that are similar in their sequence (25%) and that can bind to TβRI and TβRII. TRAP1 is supposed to be implicated into the recruitment of Smad4 upon ligand binding via direct

interaction, thus facilitating formation of heteromeric Smad complexes (Wurthner et al, 2001). TLP, in contrast, is a protein that seems to have important functions in specifying TGFβ signaling responses by its ability to preferentially activate Smad3- and inhibit Smad2 dependent signaling (Felici et al, 2003).

**Dab2**, is an adaptor molecule that constitutively interacts with TβRI, which is indispensable for active signaling by functioning as Smad2/3 adaptor (Hocevar et al, 2001). Together in complex with AP2, it can also favor receptor endocytosis to clathrin-coated vesicles (Penheiter et al, 2010).

The molecular chaperon **heat shock protein HSP90** is binding to the TβRI/II complex, to protect it from Smad7/Smurf binding and the following receptor degradation (Wrighton et al, 2008).

The metalloprotease **ADAM12** facilitates signaling by inhibiting Smad7 association with the receptor (Atfi et al, 2007).

Other proteins are important for the mediation of crosstalk with other signal transduction pathways (see Chapter 3.3.) or to induce non-canonical signal transduction pathways, like TGF β-activated protein kinase TAK1 (Yamaguchi et al, 1995) that mediates TGFβ-induced MAPK activation of p38 and JNK, or Daxx, that induces apoptosis by favoring JNK activation (Perlman et al, 2001). The cell polarity protein Par6 can be directly phosphorylated by TβRII to promote RhoA degradation by the Smurfs (Ozdamar et al, 2005), and the scaffolding protein SHC1 that binds to TβRII and gets phosphorylated by RI leads to MAPK activation (Lee et al, 2007). Further positive regulating proteins are explained in Table 2.

### 3.2.4 Regulation of Smad activity

The Smads are also an important object of positive and negative regulation of TGFβ signaling either through modifications or complex formation with proteins.

*Regulation by covalent modifications*

Despite the classical Smad phosphorylation by the type I receptors, phosphorylation on the SXS motif can be carried out by other kinases like RTKs (de Caestecker et al, 1998) or the

kinase Mps1 (Zhu et al, 2007) , which were reported to induce a ligand-independent activation of the Smads. The Smad linker region is another part of the molecule that can be subject to activity modulating phosphorylations. ERK (Kretzschmar et al, 1999) and the $Ca^{2+}$ /calmodulin-dependent protein (CaM) kinase II (Wicks et al, 2000) -mediated phosphorylations negatively regulate the Smads, whilst via JNK-mediated phosphorylation contributes to Smad activity (Brown et al, 1999b). The cyclin-dependent kinase CDK 2/4- (Matsuura et al, 2004) or CDK 8/9- (Alarcon et al, 2009) dependent phosphorylation in the linker region can either inhibit (CDK 2/4) or enhance Smad transcription activity (CDK 8/9).
Also dephosphorylation is an important step for modifying Smad activity: The phosphatases PPM1A (Lin et al, 2006) or PP2a dephosphorylate serines in the C-terminal SXS motif (Heikkinen et al, 2010), while small C-terminal domain phosphatases (SCPs) act on linker phosphorylations (Sapkota et al, 2006; Wrighton et al, 2006).
Similar to the receptors, polyubiquitylation of the R-Smads by different E3 ligases like Smurf2 (Zhang et al, 2001), ROC1 (Fukuchi et al, 2001), WWP1 (Komuro et al, 2004) and NEDD4 (Kuratomi et al, 2005) can trigger proteasomal degradation. A monoubiquitination of R-Smads by Itch/AIP4 that interacts with the receptor TβRI increases Smad activity (Bai et al, 2004). In contrast, monoubiquitination of Smad4 is supposed to promote their nuclear export (Moren et al, 2003). Smad3-Sumoylation by PIAS is another possibility for negative regulation by targeting Smads for nuclear export (Lin et al, 2006). 4

*Regulation by interacting proteins*
(I) NEGATIVE REGULATORS
**TMEPAI** was found to be a TGFβ-induced negative regulator of Smads by sequestering them in the cytoplasm through direct binding and inhibition of complex formation with SARA, an endosomal protein (Watanabe et al, 2010).

**c-Ski and SnoN**, part of the family of the proto-oncogene of the Ski family, function as inhibitors of Smad-dependent gene transcription by impairing the interaction of Smad4 and the R-Smads in the cytoplasm (Ferrand et al, 2010; Krakowski et al, 2005; Prunier et al, 2003) or upon the recruitment of co-repressors in the nucleus (Sun et al, 1999). During signaling, this negative regulation is abandoned by either Smurf2- or APC-mediated degradation of Ski and SnoN (Bonni et al, 2001; Stroschein et al, 2001).

**TGIF**, a homeobox transcription factor competes with p300/CBP to R-Smad binding and also recruits repressors and HDACs (Wotton et al, 1999a; Wotton et al, 1999b). TGIF is additionally implicated in a negative crosstalk regulation: an ERK-triggered activating phosphorylation of TGIF can negatively regulate Smad-dependent transcription (Lo et al, 2001).

(II) POSITIVE REGULATORS

**SARA** is an endosomally (EEA1 positive endosomes) located FYVE domain protein, which functions as anchor between Smads and the receptor, by having both, a Smad-binding-domain (SBD) and an receptor-interacting motif. It is described to induce proximity of R-Smads and the receptor and to be necessary for their phosphorylation by the TβRI (Tsukazaki et al, 1998). Further studies showed, that SARA might be dispensable for Smad3 signaling and only essential for Smad2-dependent signaling (Bakkebo et al, 2012; Goto et al, 2001; Lu et al, 2002).

Other proteins, such as **Hrs** (Miura et al, 2000) and ***cPML***, the cytoplasmic form of the promyelocyticleukemia tumor suppressor, (Lin et al, 2004) act as enhancer of SARA activity.

**Axin1,** an RGS/DIX domain protein, interacts with the MH2 domain of Smad3, possibly also Smad2 to facilitate signaling (Furuhashi et al, 2001). Further evidence exists that it also promotes TGF signaling by negatively regulating Smad7 through inducing its degradation via the E3 ligase Arkadia (Liu et al, 2006) (Koinuma et al, 2003).

Other interacting proteins are summarized in the following tables 2 and 3 and for interaction with transcription factors on the nuclear levels, further information can be found in (Feng & Derynck, 2005)

**Table 3 Proteins negatively regulating TGFβ signaling by receptor- or Smad interaction**

| Proteinname | Target | Proteintype | Mode Of Action | Reference |
|---|---|---|---|---|
| **Receptor interacting** | | | | |
| Bambi | RI | Decoy receptor | Competes for BMP binding and formation of nonfunctional RII complexes | Onichtchouk et al., 1999 |

| | | | | |
|---|---|---|---|---|
| c-Ski | RI | Adaptor protein | Promotes formation of nonfunctional receptor/R-Smad complexes | Ferrand et al. 2010 |
| Dpr2 | RI | Adaptor protein | Triggers lysosomal degradation of receptor | Su et al., 2007b |
| DRAK2 | RI | Kinase | Attenuation of TβRI activity | Yang et al., 2012 |
| ETV6-NTRK3 | RII | chimeric oncoprotein | Prevents interaction with of RII with RI | Jin et al., 2005 |
| FKBP12 | RI | Peptidyl-prolyl-isomerase | Prevents ligand-independent RI activation | Wang & Donahoe, 2004 |
| PP1c | RI | Phosphatase | RI dephosphorylation | Shi et al., 2004 |
| PP2a | RI | Phosphatase | RI dephosphorylation | Griswold-Prenner et al., 1998 |
| Smad 6/7 | RI | I-Smad | Competes with R-Smads, mediates receptor ubiquitylation and dephosphorylation | Nakao et al., 1997, Kavsak et al. 2001 |
| Smurf1/2 | RI/RII | Ubiquitinligase | Ubiquitin-mediated proteasomal degradation | Kavsak et al. 2001 |
| STRAP | RI/RII | WD 40 adaptor protein | Potentiates Smad7 effects and inhibits R-Smads | Datta et al. 1998 |
| TACE/ ADAM17 | RI | Metalloprotease | Shedding of TβRI extracellular domain | Liu et al., 2009a |
| TrkC | RII | RTK | Prevents interaction of RII with RI | Jin et al., 2007 |
| WWP1 | RI/Smads | Ubiquitinligase | Ubiquitin-mediated proteasomal degradation | Komura et al., 2004 |
| YAP65 | RI/RII | SH3 adaptor protein | Increase of Smad7 association | Ferrigno et al. 2002 |
| **Smad interacting** | | | | |
| c-Ski | S2/3/4 | Adaptor protein | Impairment of heteromeric Smad complex formation | Prunier et al.,2003 |
| Erbin | S2/3 | PDZ-domain protein | Sequesters Smads in the nucleus | Dai et al.,2007 |
| NEDD4 | S2/3 | Ubiquitinligase | Ubiquitin-mediated proteasomal degradation | Kuratomi et al.,2005 |
| PIAS | S3 | Sumoligase | Nuclear export | Lin et al., 2006 |
| PP2a | S3 | Phosphatase | SXS dephosphorylation | Heikkinen et al. 2010 |
| PPM1A | S2/3 | Phosphatase | SXS dephosphorylation | Lin et al., 2006 |
| ROC1 | S2/3 | Ubiquitinligase | Ubiquitin-mediated proteasomal degradation | Fukuchi et al.,2001 |
| SCP | S2/3 | Phosphatase | Smad linker region dephosphorylation | Sapkota et al., 2006, Wrighton et al. 2006 |
| Smurf2 | S2/3 | Ubiquitinligase | Ubiquitin-mediated proteasomal degradation | Zhang et al., 2001 |

| | | | | |
|---|---|---|---|---|
| SnoN | S2/3/4 | Adaptor protein | Impairment of heteromeric Smad complex formation | Krakowski et al.,2005 |
| TGIF | S2/3/4 | Homeobox protein | Competes with Smads for transcriptional co-factor binding | Wotton et al.,1999 |
| TMEPAI | S2/3 | Adaptor protein | Competes with RI for R-Smad binding | Watanabe et al.,2010 |

**Table 4 Proteins positively regulating TGFβ signaling by receptor- or Smad interaction**

| Proteinname | Target | Proteintype | Mode Of Action | Reference |
|---|---|---|---|---|
| **Receptor interacting** | | | | |
| ADAM12 | RI | Metalloprotease | Protects from Smad7 binding | Atfi et al., 2007 |
| Dab2 | RI/Smads | Adaptor protein | Required as Smad adaptor and facilitates endosomal trafficking | Hocevar et al., 2001 Penheiter et al., 2010 |
| Daxx | RI | Death domain protein | Favors apoptosis induction | Perlman et al., 2001 |
| HSP90 | RI | Chaperone | Protects from Smad7 binding | Wrighton et al., 2008 |
| Itch-AP4 E3 | RI/Smads | E3 ligase | Associates with RI and monoubiquitinates Smads for signaling potentiation | Bai et al., 2004 |
| Par6 | RII | Adaptor protein | RhoA degradation | Ozdamar et al., 2005 |
| Shc | RII | SH2 docking protein | MAPK pathway activation (ERK) | Lee et al. 2007 |
| Src | RII | Kinase | Tyrosine phosphorylation inducing p38 activation | Galliher and Schiemann, 2007 |
| TAK1 | RI | Kinase | MAPK pathway activation (p38, JNK) | Yamaguchi et al., 1995 |
| TLP | RI/RII | Adaptor protein | Promotes Smad3 but impairs Smad2 phosphorylation | Felici et al., 2003 |
| TRAF6 | RII | Monoubiquitinase | Targets intercellular domain of RI | Mu et al. 2011 |
| TRAP1 | RI/RII | Adaptor protein | Facilitated Smad4 recruitment | Wurthner et al., 2001 |
| Ubc9 | RI | Sumoylation | Sumoylated RI and enhances Smad recruitment | Kang et al. 2008 |
| UCH37 | RI | Deubiquitinase | Reverses Smurf-mediated ubiquitination | Wicks et al., 2005 |
| USP15 | RI | Deubiquitinase | Reverses Smurf-mediated ubiquitination | Eichhorn et al., 2012 |
| XIAP | RI | Multidomain protein | Cooperates with TAK1 | Neil et al., 2009 |
| **Smad interacting** | | | | |
| Arkadia | Smad7 | Ubiquitinligase | Targets Smad7 for degradation | Koinuma et al., 2003, Liu et al. 2006 |
| Axin | Smad2/3 | RGS/Dix domain protein | Associates with Smads to facilitate signaling | Furuhashi et al., 2001 |

| | | | | |
|---|---|---|---|---|
| cPML | Smad2/3 | Adaptor protein | Enhances SARA actions on Smads, favors endosomal localisation of receptor complexes | Lin et al., 2004 |
| Elf | Smad2/3/4 | Beta spectrin | Required for Smad activation and nuclear localisation | Tang et al., 2003 |
| Hrs | Smad2/3 | FYVE domain protein | Enhances SARA actions on Smads | Miura et al., 2000 |
| JNK | Smad2/3 | Kinase | Linker region phosphorylation | Brown et al., 1999b |
| Mps1 | Smad2/3 | Kinase | SXS motif phosphorylation | Zhu et al., 2007 |
| SARA | Smad2/3 | FYVE domain protein | Favors Smad association with RI | Tsukazaki et al., 1998 |

## 3.3 Crosstalk of TGFβ signaling

Another possibility for the creation of diversity in TGFβ signaling relies on the phenomenon of crosstalk with other signal transduction pathways (for definition see Chapter 1.4.).

### 3.3.1 Non-canonical signaling of TGFβ receptors

Non-canonical signaling of the TGFβ signal transduction pathway is a Smad-independent signaling, that implies all the events, where a TGFβ ligand does not signal via the traditional pathway, but other molecules being part of another signal transduction pathway. The activation of these pathways is also often correlated with a distinct functional outcome of the ligand binding. The molecules that are interacting with the TGFβ core components have also an important impact on directing signaling into a specific direction (Zhang, 2009)

*Activation of MAP kinases*

The possibility of a signaling through MAP kinases came up with the findings that Smad KO mice or cells with Smad-activation-deficient receptors could yet evoke a cellular response to ligand binding. As signaling mediators could be identified the MAPK members JNK, which mediates the TGFβ-dependent synthesis of extracellular matrix (Atfi et al, 1997; Hocevar et al, 1999) and p38 (Hanafusa et al, 1999; Yu et al, 2002) that is responsible for inducing TGFβ-mediated apoptosis. The TβRI interacting proteins TAK1 and TAB, a MAP3K and its activator respectively, have been found to be responsible for converting the ligand binding into the activation of the MAP kinases JNK and p38 (Hanafusa et al, 1999; Shibuya et al, 1996) independent of receptor kinase activity (Sorrentino et al, 2008). Additional regulators

that are essential for the induction of apoptosis of the complex are the TβRI-interacting XIAP (Yamaguchi et al, 1999) and the TβRII-interacting Daxx (Perlman et al, 2001).
It has also been shown, that TGFβ signals can lead to activation of the MAP kinase ERK by two different mechanisms: One mechanism seems to involve a transcriptional regulation occurring only late after stimulation by regulating the expression of Ras-like protein RLP, a receptor binding protein that favors ERK activation (Piek et al, 2004). Another mechanism is a short-term response, which is accomplished by the adaptor protein SHC1 that binds through its SH2 domain to a p-Tyr site in TβRII that, after being phosphorylated by the TβRII, can recruit other proteins that activate ERK by phosphorylation. This direct activation seems to be important in the process of epithelial-to-mesenchymal-transition.

*Activation of Rho GTPases*
An effect of the Rho family of small GTPases RhoA, Cdc42 or Rac1 by TGFβ has also been reported (Bhowmick et al, 2001; Edlund et al, 2002). A mechanism, implicating Par6 binding to the TβRII and subsequent Par6 phosphorylation seems to be important for the induction of RhoA degradation, allowing membrane ruffling and lamellipodia formation through Rac1 and Cdc42 during epithelial-to-mesenchymal transition (see Chapter 3.5.1).

*Activation of others pathways*
Further reports could show, that TGFβ signaling can also imply activation of NFκB that is phosphorylated by TAK1. PI3K activation occurs via direct association with the receptor complex and a following Akt phosphorylation (Bakin et al, 2000).

### 3.3.2 Crosstalk of TGFβ signaling with other signal transduction pathways

The TGFβ signaling pathway can be the object of activity modulation in a synergistic or antagonist fashion through the crosstalk with other signal transduction pathways (Guo & Wang, 2009).

*Crosstalk with MAPK pathways*
One of the most important crosstalk occurs upon interplay with the MAP kinase ERK. The basis is, that both evoke opposed responses in epithelial cells (TGFβ: cytostatis vs. Erk: proliferation), leading to antagonizing effects of the EGF-Ras-Erk axe on TGFβ signaling. Activated Erk can phosphorylate Smads in their linker region, attenuating nuclear import, thus

inhibiting Smad-dependent transcription (Kretzschmar et al, 1999). Ras was also described to decrease Smad4 protein stability (Saha et al, 2001), which can also be maintained by JNK/p38 in cancer cells (Liang et al, 2004). In contrast, during processes like epithelial-to-mesenchymal transition, synergistic effects with Ras-Erk signaling have been observed. TGFβ can elicit an increase in cytokine secretion like PDGF. A phosphorylation of R-Smads in their SxS motif induced by RTKs has been be detected (de Caestecker et al, 1998).
Other MAP kinases, with a role in growth inhibition as apoptosis have also been described to affect Smad activity. On one hand, JNK-mediated phosphorylation of Smad3 in the linker region can have enhancing effects (Brown et al, 1999b), while the JNK effector c-Jun blocks Smad activity (Dennler et al, 2000; Pessah et al, 2001).

*Crosstalk with the PI3K/Akt pathway*

Different activators of the PI3 Kinase like insulin and IGF can decrease TGFβ-dependent cytostatic effects by mainly targeting Smad3 (Chen et al, 1998). The discovery of an inhibitory interaction of Akt with Smad3 further supports this negative interplay of both pathways (Conery et al, 2004; Remy et al, 2004). PTEN, a lipid phosphatase that is a negative regulator of the PI3K pathway, was described to be repressed by TGFβ signaling (Chow et al, 2007).

*Crosstalk in embryonal development: Wnt, hedgehog and notch*

Due to the effects of TGFβ on embryonal development, crosstalk occurs with other pathways having an important role in development. The most important is the Wnt pathway, composed of Wnt, a lipid ligand, with its GPCR frizzled, that regulates β-catenin activity and nuclear translocation. During development a reciprocal regulation of ligand concentration of Wnt and TGFβ has been observed, and synergistic effects can be obtained through gene transcription-mediated by complexes of R-Smad/β-catenin/Lef. Furthermore, interactions between TGFβ and the hedgehog- and notch pathways seem to be critical for accurate embryonic development (Wu & Hill, 2009).

*Crosstalk with interleukins (IL), TNF and interferon (IFN)-γ pathways*

Acting also on the immune systems, crosstalk with pathways responsible for the immune reaction of a cell, like IL-pathways, the TNFα/β or IFNγ pathway are also occurring (Li et al, 2006).

*Crosstalk on a nuclear level*

In the nucleus, crosstalk has been observed with the AP-1 transcription factor complex, required for apoptosis (Schuster & Krieglstein, 2002). Another important crosstalk that synergizes the cytostatic effects of TGFβ signaling occurs due to the interplay with tumor suppressor p53 related signaling (Atfi & Baron, 2008).

*Pathways involving Smad phosphorylation*

As for example the crosstalk with MAP kinases has shown before, the Smads and their multiple phosphorylation sites are a key target for integrating signals of other pathways. Further kinases were identified to modulate Smad activity by their phosphorylation: a negatively regulating phosphorylation can be exerted by the calcium-calmodulin dependent-kinase. (Wicks et al, 2000), and a PKC phosphorylation on the MH1 domain has a negative effect by abolishing DNA binding (Yakymovych et al, 2001). Also the cyclin-dependent-kinases CDK2 and CDK4 phosphorylate Smad and inhibit Smad-dependent gene transcription and thereby their cytostatic effects (Matsuura et al, 2004). In addition, also the casein kinase I, glycogen synthase kinase GSK3 or GRK2 (see below) can modify Smad activity through phosphorylation (Wrighton et al, 2009).

#### 3.3.2.1 Crosstalk with GPCR signaling and transactivation by GPCRs

So far, only little information exists concerning the phenomenon of crosstalk and transactivation of TGFβ signaling pathways and GPCR-mediated signaling.

*Crosstalk between GPCRs and TGFβ signaling*

Some work could provide evidence for the existence of crosstalk between GPCRs and TGFβ signaling. The GPCR regulating protein β-arrestin 2 has also been linked to the modulation of TGFβ signaling: the association of β-arrestin 2 with the TβRIII betaglycan can induce internalization of the TβRII/TβRIII complex and subsequently downregulate TGFβ signaling (Chen et al, 2003). Another work reported also its direct association with the TβRII, even in the absence of the RIII, that might have promoting effects in directing signaling towards non-canonical pathways, like p38-induced apoptosis (McLean et al, 2013). This goes along with the role of β-arrestins in GPCR signaling by favoring MAP kinase activation (see Chapter 1.3.). Furthermore, evidence is provided for TGFβ signaling regulating GPCR signaling proteins, such as GRK2 for being a TGFβ response gene. As a result of its TGFβ-stimulated

expression, GRK2 associates with and phosphorylates the linker region of Smads, hence inhibiting their nuclear translocation (Ho et al, 2005). So far, there has been no proof, whether GRK2 might also have an effect on receptor phosphorylation, what might trigger the described β-arrestin 2 association with the receptors.

Besides, studies have shown, that the Serotonin receptors 5-$HT_{1B/D}$ can induce, after stimulation with serotonin, a phosphorylation of Smad1, 5 and 8, which normally signal through BMP ligand (Liu et al, 2009b), in a RhoA Kinase (ROCK) -dependent manner. An example for TGFβ-induced regulation of GPCRs was shown for the TGFβ-induced delay of neurokinin receptor internalization in T-cells leading to prolonged signaling (Beinborn et al, 2010).

*Receptor transactivation*

Compared to crosstalk, transactivation is a more limited term, to describe the interplay of signaling pathways. While a crosstalk also implies *de novo* gene transcription or signal-mediator-dependent responses, a transactivation is "where one receptor activates a heterologous receptor" (Wetzker & Bohmer, 2003), meaning an immediate effect on the receptor level. Compared to the big amount of sources describing an interplay between GPCR signaling and RTK-triggered pathways (see Chapter 1.3.), just recently the group of Peter Little demonstrated an association of TGFβ- and GPCR-mediated signaling pathways on the receptor level, what led to the expansion of the transactivation concept of GPCR towards serine/threonine kinases (Burch et al, 2012). They have shown, both for endothelin and thrombin GPCRs, that ligand binding induced a transactivation of the TGFβ signaling pathway via Smad phosphorylation. The transmission of the signal from the GPCR to the TGFβ receptor was proven by inhibition of the TβRI kinase activity that abolished these events (Burch et al, 2010; Little et al, 2010). But the exact mechanisms underlying this transactivation remain unknown and so far, their studies form the only example for GPCR-ligand-induced transactivation of the TGFβ receptor.

## 3.4 TGFβ signaling in physiology and pathophysiology

TGFβ ligands are multifunctional molecules that are known to regulate many cellular processes like proliferation, cell fate determination, differentiation, motility, adhesion, that are essential for the development and maintenance of tissue and organism homeostasis. TGFβ cytokines act mainly in an autocrine or paracrine manner and virtually all cells can respond to

ligand stimulation. In contrast, the physiological effects strongly dependent on the cellular context and the expression pattern of different molecules implicated in TGFβ signaling, leading to the variety of possible TGFβ signaling outcomes that can be completely opposite dependent on the physiologic environment. These pleiotropic actions of TGFβ also explain its large implication in human disease (Massague, 2012).

### 3.4.1 Biological actions of TGFβ signaling

TGFβ ligands have been found to be implicated in a huge variety of processes in organisms, the most important ones will be highlighted in the following and are summarized in Figure 20:

#### 3.4.1.1 Cytostasis and apoptosis

In most of the cell types, the cellular response to TGFβ is an inhibition of cell proliferation. This can observed in epithelial, endothelial, hematopoietic and immune cells, while in mesenchymal cells as fibroblasts, the opposite effect can take place. On the molecular level, the TGFβ-induced cytostatis is the result of activation and repression of gene expression of important players of cellular proliferation in the G1 phase of the cell cycle (see Figure 19). An early TGFβ response effect is the repression of the expression of the proto-oncogene c-myc (Alexandrow et al, 1995). This is accompanied by a loss of the repressive actions of myc on cell cycle inhibitors p15INk4b and p21kip. TGFβ promotes additionally the expression of p15Ink4b that inhibits cyclin D/CDK4 and CDK6 in early G1 stage and p21Kip that acts on cyclin E/A-CDK2 activity. This repression-activation cascade is the result of concerting actions: in order to repress myc transcription, a preformed cytoplasmic complex composed of the transcription factors E2F4/5, DP1 and p107 exists, which translocates with activated Smads to the nucleus, binds to the myc promoter and represses it expression. Myc itself is a repressor of p15Ink4b. By inhibiting myc transcription, the formation of the repressive complex with the miz protein is abolished, conducting to the feedback activation of p15Ink4b (Seoane et al, 2001; Staller et al, 2001).

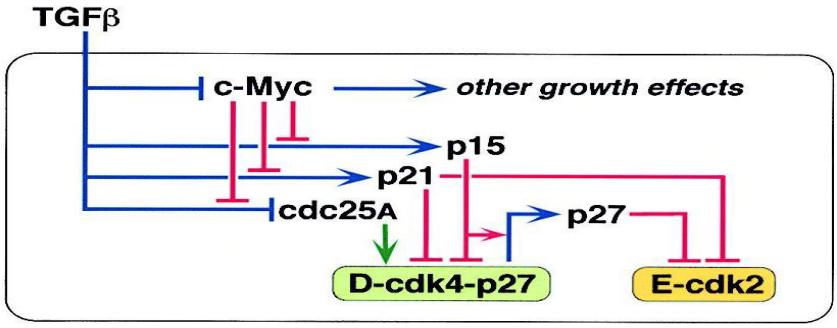



**Figure 19 Mechanism of TGFβ-induced cell cycle arrest (Massague et al, 2000)**

TGFβ induces a repression of the growth promoting protein c-myc. This in turn leads to an arrest of the repressive actions of myc on the cell cycle inhibitors p15, p21 and cdc25A. Their expression is also stimulated by TGFβ signaling. Their activity leads the repression of the cell cycle promoting cycline dependent kinases CDK2 and CDK4, thus explaining the cytostatic effects of TGFβ.

Other events that play a role in exerting cytostatic effects are the repression of the ID proteins (ID1, ID2, ID3), performed by a complex of Smads with the selfenabling ATF3 protein (Kang et al, 2003a). Also the interaction with the transcription factor Runx3 and the crosstalk with p53 participate in growth control by TGFβ (Pardali & Moustakas, 2007).

Under certain conditions, TGFβ can not only induce a block of the cell cycle, but can also directly induce apoptosis. So far, several mechanisms have been discovered that propose an involvement of caspases, Bcl-2 family members like the protein BIM, and death-associated protein kinase (DAPK) triggering the mitochondria associated pathway of apoptosis (Chen & Chang, 1997; Jang et al, 2002; Ohgushi et al, 2005). The TβRII-interacting protein Daxx seems to be important to direct signals towards apoptosis via JNK- and p38 activation (Perlman et al, 2001). Physiologically this induction of apoptosis occurs for example in the mammary gland size reduction after pregnancy.

#### 3.4.1.2 Development and embryonal stem-cell differentiation

TGFβ signaling is strongly implicated in the orchestration of embryogenesis and development. The occurrence of ligand gradients is fundamental for correct developments at specific stages. Different ligands are required for establishment of the left-right (nodal), correct vascular, cardiac, lung and craniofacial development (TGFβ) and the Smads have been shown to be required for the anterior-posterior axe establishment and endoderm formation (Wu & Hill, 2009).

With regards to embryonic stem cells, BMP-dependent actions promote self-renewal of stem cells by inducing a feed-forward cycle with the differentiation repressing triad of the transcription factors OCT4/SOX2/NANOG (Chen et al, 2008). In contrast, Smad2 can drive me endodermal differentiation by activating transcription of genes with an ARE motif, recognized by a Tripartite motif-containing 33 (TRIM33)-Smad2 complex (Xi et al, 2011). In progenitor cells, the synergistic action of identity factors and Smads on gene expression implements differentiation programs like for myoblast (MYOD1), mesenchymal and lymphoid (PU.1), myeloid (C/EBPα) and erythroid (GATA) progenitors (Watabe & Miyazono, 2009).

#### 3.4.1.3 Epithelial-to-mesenchymal transition (EMT)

EMT is a process mainly driven by TGFβ, meaning the switch from epithelial cells into a migratory mesenchymal phenotype like in (myo)fibroblasts. During development, it is necessary for gastrulation, embryonic tissue formation and in adult tissues required for regenerative processes. The molecular events underlying EMT are on the one hand a loss of cell polarity, which is mediated by Par6 that interacts with and gets phosphorylated by TβRII and then induces RhoA degradation (Ozdamar et al, 2005). On the other hand, the Smads also induce the expression of the selfenabling transcriptional repressors Snail and Slug that together repress the expression of E-cadherin, a marker of epithelial cells and favor the expression of N-cadherin, marker of invading and migrating cells. Additionally, a preceding crosstalk with Wnt signaling activity creates an environment favoring TGFβ to induce EMT instead of growth arrest. Also other crosstalks are implicated to realize a switch from the proapoptotic Smad-pathway to pathways that favor migration (Heldin et al, 2009). Besides the beneficial effects of EMT in tissue repair and wound healing, EMT is a process crucial for cancer progression and occurs pathologically in fibrosis (see below).

#### 3.4.1.4 Regulation of extracellular matrix (ECM)

The ECM is surrounding the cells, forms the major component of the connective tissue and is composed of characteristic proteins as collagen, elastin, fibrillin, fibronectin, lamin and proteoglycans. A lot of genes encoding for ECM components are found to be regulated by Smads, as collagens (Chen et al, 1999), making TGFβ a critical regulator of ECM synthesis (Verrecchia & Mauviel, 2007). The plasminogen-activator-inhibitor 1 (PAI-1), is a TGFβ

response gene important in maintenance of ECM and inhibition of fibrinolysis (Laschinger et al, 1991). This TGFβ-stimulated ECM formation is of big importance in wound healing (O'Kane & Ferguson, 1997), but can turn out negatively by excessive signaling and consequently lead to fibrosis.

#### 3.4.1.5 Angiogenesis

Though having a repressive effect on proliferation in endothelial cells, TGFβ acts as potential angiogenic factor by favoring endothelial cell migration (Roberts et al, 1986). But things are much more complex, because TGFβ effects seem to depend on ligand concentration and the receptor they choose for signaling: in endothelial cells, TGFβ can signal either via the classic TβRI with its anti-proliferative actions, while signaling through ALK1 is associated with pro-proliferative actions. The endothelial cells are an example where the balance of receptor utilization is important for the functional outcome (Pardali et al, 2010). In order to promote angiogenesis, TGFβ has been found to induce transcription of angiogenesis stimulation vascular endothelial growth factor VEGF (Ferrari et al, 2009) .

#### 3.4.1.6 Hematopoiesis

TGFβ acts as potent inhibitor of hematopoietic stem cell proliferation. The TGFβ-1 ligand inhibits hematopoietic stem cell (HSC) proliferation, leaves them in a quiescent state and downregulates receptors for hematopoietic cytokines that would promote their differentiation (Yamazaki et al, 2009). This in turn also affects immune cell development that arise from HSCs.

#### 3.4.1.7 Bone formation

Bone is the tissue that gives us mechanical support and organ protection, regulates calcium levels and hematopoiesis. The bone is composed of osteoblasts and osteoclasts. The role of TGFβ1 as predominantly expressed isoform lies in osteoblast progenitor recruitment, stimulation of proliferation and differentiation and inhibition of apoptosis, as revealed with TGFβ KO mice that lead to reduced bone growth and mineralization (Janssens et al, 2005). Also the BMP ligand has important functions in bone homeostasis and its deletion leads to impairment in bone formation.

#### 3.4.1.8 Reproduction

TGFβ signaling also has critical functions in the female reproductive system: it regulates ovarian follicle development, gonadotropin receptor expression, oocyte maturation, ovulation and luteinization (Abassi & Vuori, 2002). These actions are mainly mediated by the activin-inhibin-follistatin system: activin is the ligand, while inhibin functions as a decoy ligand and follistatin directly sequesters activin through direct binding. An important role in controlling reproduction role might also be assigned to TGFβ signaling in the tanycytes, the brain cells surrounding the 3$^{rd}$ ventricle that are implicated in the regulating of reproduction (Bouret et al, 2004; Bouret et al, 2002; Prevot et al, 2000).

#### 3.4.1.9 Immune system

The importance of TGFβ signaling in the immune system becomes obvious with regards to TGFβ ligand KO mice, displaying a strong immune deregulation with hyperactivity of the immune system and infiltration of organs with immune cells, showing that TGFβ is an important immunosuppressant agent. It is acting negatively on the proliferation, differentiation and activation of T cells, B cells, natural killer (NK) cells, monocytes, macrophages, neutrophils and eosinophils. In contrast, it can exert pro-inflammatory effects, by directing immune cells into the development of a secretory phenotype, leading to the secretion of IL-6, IL-11 (Li et al, 2006).

#### 3.4.1.10 Nervous system

TGFβ ligands are implicated in the establishment of synaptic plasticity and play a role in cognition and behavior. They are supposed to have neurotrophic and neuroprotective roles and are involved in excitatory and inhibitory neurotransmission. Furthermore, TGFβ influences neurite outgrowth and synaptogenesis (Krieglstein et al, 2011). TGFβ mediates axon specification during axon development in a Par6-dependent manner (Yi et al, 2010).

#### 3.4.1.11 Energy homeostasis

Recently actions of TGFβ in energy homeostasis were proposed, by showing a role for Smad3 in inhibiting the transformation of white adipose into adult brown adipose tissue through negative regulation of expression of markers of the brown adipose tissue (Yadav et al, 2011).

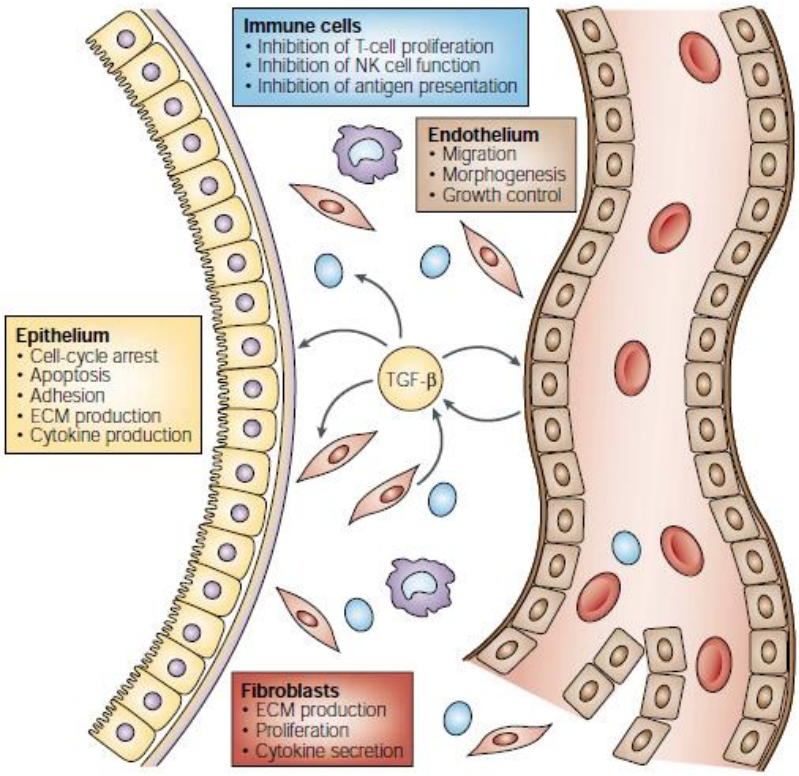



**Figure 20 Effects of TGFβ on different cell types (Siegel & Massague, 2003)**

The effects of TGFβ are cell type specific. In epithelial cells (yellow), mainly cytostatic and apoptotic actions are observed and adhesion and ECM production are supported. In endothelial cells (brown) their migration is promoted. Immune cells (blue) mainly receive proliferation- and differentiation-inhibitory signals. In fibroblasts (red), TGFβ can stimulate proliferation and ECM production.

### 3.4.2 Pathophysiological implication of TGFβ signaling

Being such a pleiotropic molecule, malfunctioning of the TGFβ pathway can result in numerous diseases. Germline or somatic mutations and alterations in expression of signaling components can result in abnormal physiological function. Dissecting the effects of TGFβ signaling in disease helps to reveal and understand the physiological functions and their analysis can lead to identification of underlying mechanisms and the development of appropriate therapeutic strategies (Gordon & Blobe, 2008).

*Cardiovascular processes and cardiovascular disease*

TGFβ ligands are implicated in cardiac development and angiogenesis, as supported by KO mice of the TGFβ ligand (1,2,3) that show ventricular septum defects, myocardial thinning, double outlet right ventricles, failed coronary vessel and epicardial development. Smad4 KO in the heart leads to a hypocellular myocardial wall defect (Bobik, 2006). Disorders that were

found to be associated with TGFβ are *hereditary hemorrhagic teleangiectasia*, with a vascular dysplasia that results in teleangiecstasia and arteriovenous malformation, based on inactivating mutations in the type I receptor ALK1 (Abdalla & Letarte, 2006). Impairment of receptor activity was also found to take place in aorta diseases or pulmonary hypertension. Loss of TGFβ signaling activity is associated with a higher risk to develop pre-eclampsia during pregnancy and atherosclerosis. In contrast, hypertension seems to be correlated to an increase of TGFβ levels, which might also rely on the effects by the blood pressure regulating actions of the renin-angiotensin-system to upregulate TGFβ expression (Wolf, 2006).

*Connective tissue diseases*

By positively regulating the production of ECM molecules and being dependent on ECM molecules for its activation, TGFβ signaling malfunction can lead to different disease phenotypes.

The Marfan Syndrome is a hereditary disorder, displaying a mutation of the ECM protein fibrillin-1, that leads to altered anchoring of inactive TGFβ ligand and reduction in TGFβ release, resulting phenotypically in aneurysms, aortic dissections and skeletal manifestations (Lindsay & Dietz, 2011)

Another disorder that is associated with TGFβ actions on ECM is fibrosis. Fibrosis occurs when the positive effects of TGFβ-mediated disposition of ECM molecules by myofibroblasts, for example during wound healing, lose their balance and become excessive. In combination with the pro-proliferative effect of TGFβ on myofibroblasts, an accumulation of fibroblast that produce too many ECM molecules is the consequence. A specific type of fibrosis is restenosis, a fibrotic response to revascularization therapies, where TGFβ can have a contributing effect, but the increased ECM production is often leading to complications (Verrecchia & Mauviel, 2007).

*Other diseases*

The other important functions of TGFβ in different organs listed in 3.4.1. can give rise to other diseases associated with TGFβ signaling.

TGFβ and BMP ligands are indispensable for a proper bone homeostasis. Therefore, defective TGFβ signaling can lead to diseases as osteoporosis (Su et al, 2007a). In contrast, hyperactivity of the signaling pathway due to an amino acid substitution mutation in the GS domain of the ACVR1 type I receptor, which leads to its constitutive activation causes

increased bone mass and ossification as in *fibrodysplasia ossificans progressiva* (Groppe et al, 2007; Groppe et al, 2011)

A dysfunction of TGFβ can also lead to altered development of reproductive organs, as shown for impaired testis or female maturation. A non-functional MIS ligand or its corresponding receptor causes a loss of regression of the Mullerian duct, leading to male persons having both male and female reproductive system, resulting in complete infertility, the persistent Mullerian duct syndrome (Josso et al, 2005).

A failure of appropriate signaling during embryogenesis at the stages of blastula formation, gastrulation or organ development can either have lethal consequences, lead to mild disorders as the cleft palate, where palate fails to undergo EMT, or cause severe deformations, as the inversion of human organ asymmetry (*situs inversus* and *situs ambiguus*), caused by mutations of the ligands important for their establishment as the TGF superfamily members Nodal, GDF and Lefty (Lowe et al, 1996).

Due to its huge immunosuppressant actions, defective TGFβ signaling can be the reason for diverse autoimmune diseases (Li et al, 2006).

Alterations of TGFβ signaling have also been associated with neurological and psychiatric disorders as parkinson, multiple sclerosis, schizophrenia and Alzheimer disease (Vivien & Ali, 2006).

#### 3.4.2.1 TGFβ signaling in cancer

One of the cruelest diseases and one of the main challenges of modern civilization is cancer. Cancer is the occurrence of malignant neoplastic transformations in one organ that can lose growth control and invade throughout the whole body. This is affecting proper function of the organism and often leads to death of the concerned individuals. Cancer development, progression and therapy are among the most researched fields and the findings helped to reveal the unique properties of cancer cells ("hallmarks of cancer" (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011)).

With its growth inhibitory function, the TGFβ signaling pathway is supposed to be an important actor in cancer development and progression. The huge amount of data that correlate malfunction or inactivation of TGFβ signaling components as a result of mutations found in tumor cells, is coherent with its important tumorsuppressing role. But the actions of TGFβ are paradox: while in early stages, TGFβ functions as a tumorsuppressor, in late stages, TGFβ signaling can enhance cancer progression and metastasis via its ability to support

cellular migration and EMT. A cancer must overcome the barrier established by TGFβ signaling in order to develop, but then it can benefit of TGFβ signals in progressing and spreading. Thus, maintenance of homeostasis in TGFβ signaling is vital for inhibition of cancer development. Consequently, the understanding of TGFβ signaling is a notable research objective to help to increase the knowledge about cancer and to identify possible therapeutic targets, either to prevent cancer onset in early stages or to limit its progression.

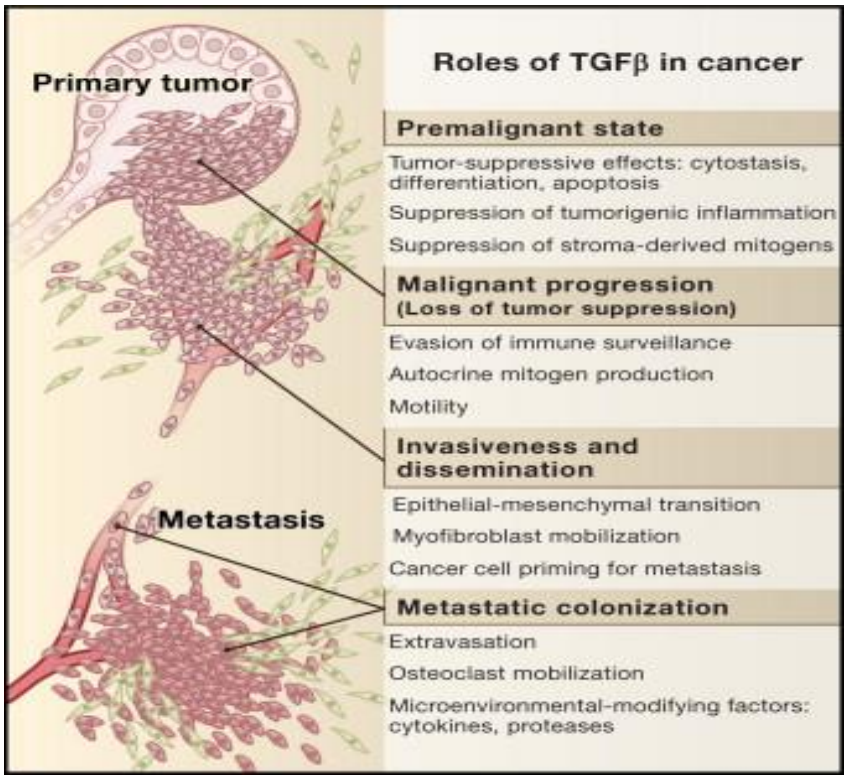



**Figure 21 Pleiotropic actions of TGFβ in cancer (Massague, 2008)**

During cancer development, TGFβ has multiple roles. While acting as a tumorsuppressor in early stages, through its cytostatic actions, malignant tumors can profit from TGFβ signaling with its abilities to promote migration, suppress immune response and to induce EMT to favor invasion and metatasis.

### A - Tumor suppressing actions of TGFβ signaling

In early stage tumors, TGFβ exhibits growth inhibitory function and protects against tumor progression through autocrine and paracrine mechanisms. In autocrine, cell autonomous mechanisms of suppression, TGFβ signaling induces its cytostatic program by myc-repression, p15 and p21 activation and ID1 downregulation. Furthermore, the induction of cellular differentiation for certain cell lines can have, dependent on cancer type and cell,

cancer-limiting effects. In conditions of excessive proliferation, as we find them in cancer with strong proliferating phenotypes e.g. as a result of Ras mutation, TGFβ can even induce apoptosis of cells in order to eliminate these deviant cells (Massague et al, 2000).
In a paracrine manner, the interplay of tumor and stroma can also generate suppressing effects, which emphasizes the influence of cellular communication of the tumor environment on cancer development. The tumor adjacent cells secret TGFβ in order to protect the tumor development by inducing cytostasis and repression of the expression of fibroblast derived mitogens (Massague, 2008).

*Loss of TGFβ dependent tumor suppression*

Cancer cells are genetically unstable and selective pressure gives advantage to those cells, who gained a surviving function due to the occurred modifications in their DNA. TGFβ signaling pathway related genes display an important target for a cancer to gain forces, explaining the high amount of genetically based dysfunction of the TGFβ signaling in cancer samples. Growing significance concerns the incidence of epigenetic changes that affect expression levels in cancer (Inman, 2011). The loss of TGFβ tumorsuppressing effects can have two distinct reasons:

(1) Loss or inactivation of core components and signaling

This type of defective TGFβ signaling is mainly found in colorectal, pancreatic, ovarian, gastric, head & neck cancer and elicits the complete loss of tumorsuppressing effects. Evidence was obtained from transgenic KO mouse models that display an increase of tumor progression (Pardali & Moustakas, 2007). Also human studies revealing inactivating germline and somatic mutations in TGFβ signaling core components show the connection of TGFβ and its tumorsuppressing role in cancer. Concerning the receptors, a frequent TβRII microsatellite instability in a polyadenine region, that is leading to expression of a non-functional receptor, can, as germline mutation lead to the hereditary non-polyposis colorectal cancer (HNPCC), or as somatic mutation to gastric tumors (Chang et al, 1997). Also other inactivating mutations in TβRI and TβRII or a decrease in their expression was found to be associated with lung, ovarian or pancreatic cancer. Additionally, Smad mutations are often referred to be tumor promoting. A Smad4 deletion, loss of heterozygocity or inactivating mutations are found in 50% of pancreatic carcinomas (Hahn et al, 1996; Schneider & Schmid, 2003) and other

intestinal forms, as colon carcinoma. In contrast, the R-Smad mutations are less frequent in cancer (Massague et al, 2000).

(2) Loss or disabling of the tumor suppressor arm

Another possibility is only the loss of the tumor suppressing arm of TGFβ signaling. This form is mainly observed in breast and prostate cancer, glioma, melanoma and hematopoietic neoplasias. A partial loss of growth inhibiting actions of TGFβ signaling often occurs beyond the core signaling pathway of ligand, receptor and Smads, and enables a cell to still benefit from the intact core parts of TGFβ signaling. This gain of function transforms TGFβ to a tumor promoting factor. Thus, a tumor can take advantage of pro-tumorigenic actions of TGFβ and incorporate them to increase its proliferative, metastatic and invasive capacities.

One reason for this transformation lies in the affection of TGFβ signaling regulating proteins (see 1.3.3.) concerning their expression levels or activity. Negative regulators of TGFβ signaling are often proto-oncogenes and an increase in activity or expression can lead to a loss of effectiveness of TGFβ signaling, as show for DRAK2 or the Ski/SnoN complex. The other way round, TGFβ signaling enhancing molecules can function as tumorsuppressors and a decrease in their activity or expression, can lead to a loss of growth inhibition, as known for example for Runx3.

A loss of the cytostatic response can also be completely independent from TGFβ signaling molecules, but due to a deregulation of non-signaling parts (Gomis et al, 2006). Reasons can be an increased myc-expression that is minimalizing the effect of TGFβ on myc repression and consequently blocks the upregulation of cell cycle inhibitors. These cell cycle inhibitors, being TGFβ effector genes, can also be the object of genetic loss or disabling mutations. Also signaling crosstalk plays another role: an increasing amount of proteins that accomplish inhibitory phosphorylations on Smads can diminish their activity, as for example an often described Ras or CDK overexpression (Matsuura et al, 2004). Generally, an imbalance of tumorpromoting and tumorsuppressing events in tumorous tissue can lead to a weakening of the tumorsuppressing effects of TGFβ.

**B- Tumor promoting actions of TGFβ signaling**

*The paradoxon: a switch from tumor suppression to tumor promotion*

The described loss of tumor suppression is the first step to accomplish the vicious change of TGFβ from being a tumor suppressor to becoming a tumor promoter. Having lost the growth

inhibitory branch of signaling, but remaining intact in its components, a cancer cell can benefit from the TGFβ core pathway to pursue its aggressive and invasive actions. There is also correlation of TGFβ expression and severeness of tumor. And tumors, often displaying an overproduction of TGFβ for growth inhibitory purposes, can utilize this amount for their own advantage.

*TGFβ-induced secretion of mitogens*

Under certain conditions, TGFβ can also become a pro-proliferative agent, as it does in mesencyhmal and vascular smooth muscle cells. By stimulating the autocrine or paracrine production of mitogenic substances as PDGF or HGF in gliomas or liver, TGFβ can stimulate cell growth (Ikushima & Miyazono, 2010).

*Immune-suppressive actions of TGFβ*

The paracrine TGFβ actions on tumor stroma can also be advantageous for tumor progression via suppression of the immune system. This is harbored in the suppressing effect of TGFβ on hematopoietic stem cell differentiation and with regards to the tumor environment in a suppression of immune cell maturation (CD4+ and CD8+ cells) and immune cells secreting pro-apoptotic factors like dendritic cells, cytotoxic T lymphocytes and natural killer cells. This renders a tumor being partially protected from immune surveillance (Torre-Amione et al, 1990).

*TGFβ-induced renewal of tumor-initiating cells (TICs)*

Another important tumorpromoting effect of TGFβ lies in the occurrence of the cells that gain pluripotent stem-cell-like capacities during tumor progression, the tumor-initiating cells (TIC) (Iwasaki & Suda, 2009). TGFβ can promote their self-renewal and inhibit their proliferation and differentiation, what promotes the persistence of this cancer-promoting cell type. This was reported to be especially of relevance in glioma-initiating and leukemia-initiating cells (Yamazaki et al, 2009). Another contributing TGFβ-mediated action is the epithelial-to-mesenchymal-transition that breeds cells with a similar phenotype as the TICs.

*Role of epithelial-to-mesenchymal transition on tumor progression and metastasis*

A process that resembles TIC formation is the transformation of epithelial cells into mesenchymal-like cells (Mani et al, 2008). The EMT implies the creation of a motile cell

type, the myofibroblast (or "tumor associated fibroblast") with migratory and invasive capacities that can contribute to the metastatic capacities of tumors.

Metastasis is a succession of migrations in different compartments: first local invasion from epithelial cells into lymph- or blood vessels, then circulation and invasion into the metastatic site and finally adaption and growth in a new environment. For each tumor subtype, depending on their tissue of origin, exist distinct patterns and organ preferences in metastasis, as breast cancers preferentially spread into bone or lung. The survival rate of a tumor patient decreases with its associated metastasis rate. The function of TGFβ in the onset of metastasis is backed by the existing correlation of increasing TGFβ concentrations in invading tumors compared to primary ones and the association of metastasis with higher levels of TGFβ. Also mouse models with TGFβ overexpression tend to have more invasive and aggressive cancer forms (Heldin et al, 2012).

Generally, EMT is a crucial step for metastasis: the cells escape the contact with the environment by disassembly of cell-cell junctions and develop migratory abilities what allows the transformation into invasive carcinomas (Thiery, 2002; Thiery et al, 2009).

In addition to the described mechanisms, especially the p53 crosstalk (Adorno et al, 2009), epigenetic alterations, and the involvement of TACE-induced cleavage of TβRI and its translocation to the nucleus can support the TGFβ-dependent expression of genes for EMT induction (Mu et al, 2011).

Once, the motile phenotype is generated, the cells require further priming for their dispersion in the organism. A molecular target, that has been identified to be relevant for breast cancer cell priming for distant metastasis into lung is effected by Angiopoietin-4 induction (Padua & Massague, 2009). These spreading cells then need to use their invasive capacities in order to enter and colonize in the new tissue. This has been well studied for the metastasis of MDA-MB-231 breast cancer cells as well as the mechanism they use to invade into bone tissue. The cells induce the production of substances that promote osteolysis and enable their nesting in the bone (Yin et al, 1999).

*Tumor angiogenesis*

Another hallmark of cancer and necessity for survival is neoangiogenesis, providing a tumor with new blood vessel for ensuring metabolism and oxygen provide. A lot of evidence exists for correlation of angiogenesis and TGFβ levels (de Jong et al, 2001). TGFβ can also promote the expression of VEGF, an important angiogenic driving force. However, dependent on the cancer subtype, also inhibiting effects have been described.

With all these described actions of TGFβ, we can conclude that it is a main actor in cancer, with its protecting functions in early stages and its supporting actions on late stage tumors to generate an aggressive and invasive phenotype. This underlines the importance to study the signaling of TGFβ in suitable cellular models.

### 3.4.3 TGFβ signaling as therapeutic target

This multitude of implications of TGFβ signaling in physiology and pathophysiology makes clear that TGFβ is an interesting therapeutic target. It might also function as biomarker, to identify cancer stages and their malignancy, and TGFβ serum levels can serve as marker in cardiovascular diseases or preeclampsia.

The huge beneficial actions of TGFβ can claim for a curative enhancement of signaling, for example in osteoporosis, diseases with a hyperactive immune system or in early cancer stages. This can be obtained by administration of ectopic agonist or by the means of gene therapy via an increase of the expression of signaling components.

But with regards to disorders where TGFβ signaling is not desired, strategies for its inhibition are required. A lot of effort has been put into the development of antagonizing methods and they include neutralizing antibodies, ligand traps, soluble receptors that function as scavenger, receptor antagonists, antisense oligonucleotides or small molecule receptor inhibitors. Their application can be beneficial to cancer treatment, especially in the late state, the Marfan syndrome or fibrosis. Antibodies against the TβRII to treat glioblastoma are already in the clinical trial phase (Akhurst & Hata, 2012). But still being in its infancy, a further description of the TGFβ signaling pathway and the identification of more context-specific molecular targets that are part of the signaling network can improve the development of further therapeutic strategies.

# OBJECTIVES OF THIS WORK

After having gained evidence for ligand-independent functions of GPR50 within the heterodimer with the melatonin receptor $MT_1$ and additional reports on Nogo-A and TIP60, proposing that GPR50 behaves as an interacting and activity-modulating protein, our laboratory tempted to identify further putative binding partners of GPR50. Upon application of the tandem affinity purification (TAP) technique, native GPR50 complexes were purified and subsequent mass spectrometric analysis revealed several potential interaction partners. Among these proteins was the transforming growth factor β receptor type I (TβRI). After a first *in vitro* verification of this interaction, the objectives of my work where the following: a further characterization of the interaction, including revelation of the interacting domains and the establishment of an endogenous complex formation. Another important part of my work was to characterize the impact of GPR50 on TβRI-dependent signal transduction on different levels of the pathway by the use of different methods. After having deciphered GPR50's role in signal transduction, the unraveling of the underlying mechanism displayed another objective of the work. Finally, after the accomplishment of the mechanistic part, the last aim was, to establish a functional role for GPR50 in relation to TGFβ-dependent (patho)physiological effects. A first model concerned the TGFβ-signaling-deficient gastric cancer cell line SNU638 to gain further insight in signaling mechanisms and the effect of a presence of GPR50. Furthermore, we chose the MDA-MD-231 breast cancer cells as a model to study differences in the absence and presence of GPR50. The idea behind was, to determine the impact of GPR50 on TGFβ signal transduction and to correlate this to functional effects for this novel regulator of TGFβ-dependent signal transduction. Research based upon the described axis during this thesis project should enable us to gain information about how and when the complex of GPR50 and TβRI is formed and which are its consequences on signal transduction and (patho)physiological processes.

# II. RESULTS

**Article**

## Ligand-independent activation of type I TGFβ receptor (TβRI) by the orphan 7-transmembrane protein GPR50 in the absence of the TβRII

## 1. Introduction

Emerging evidence exists that the orphan 7TM protein GPR50, a member of the melatonin receptor GPCR subfamily that lost its melatonin binding ability during evolution, has ligand-independent functions. This consists in the binding of other proteins and the modulation of their activity, as it has been shown on the membrane level for heteromerisation with the $MT_1$ (Levoye et al, 2006a) and in the cytosol for Nogo-A (Grünewald et al, 2009) and TIP60 (Li et al, 2011). In order to further establish this role, we sought to identify further putative binding partners for GPR50. By the application of a method specifically established for GPCRs in our laboratory, the tandem affinity purification (TAP) assay (Daulat et al, 2007), we tempted to isolate GPR50-associated complexes spontaneously forming in HEK293 cells. One of the identified proteins is the transforming growth factor β (TGFβ) receptor type I (TβRI) serine/threonine kinase, which is part of the TGFβ signal transduction pathway.

The TGFβ superfamily of cytokines contains molecules, which are important for the maintenance of organism homeostasis as they regulate processes like cytostasis, apoptosis and cellular migration (Massagué, 2000). The TGFβ signal transduction pathway is a simple and direct pathway: the ligand binds to one membrane type II receptor, the TβRII, which recruits the TβRI into the complex and propagates the signal through an activating phosphorylation of the TβRI. The activated TβRI then transduces the signal to the R-Smads 2 and 3, which subsequently, after their phosphorylation, form a complex with Smad4, translocate to the nucleus and regulate the transcription of target genes that execute TGFβ-mediated biological responses (Shi & Massagué, 2003a).

The ubiquitous TGFβ signaling pathway is composed of a various number of ligands and different receptors that can evoke a multitude of cellular responses. This stands in contrast to the simple way of signaling flow that is mediated by a small number of different Smad proteins. Therefore, a lot of regulatory proteins acting on different levels of the pathway have been discovered in the last years. They are implicated in specifying the TGFβ signaling pathway in a context-dependent manner upon a multitude of different modes of action (Kang et al, 2009; Xu et al, 2012). In the following work, we wanted to identify the mechanism and mode of action of GPR50 on TβRI dependent signaling. Additionally, we wanted to investigate whether the two frequent variants of GPR50, the GPR50wt and the GPR50Δ4 comprising the $^{502}$Thr Thr Gly His$^{505}$ deletion in the C-terminus display any functional differences.

The first part of the work focused on the description of the interaction and its character. We used different techniques, in order to validate the interaction, as co-immunopreciptation and the BRET technique. A study in different cell types upon overexpression and endogenous levels, helped to gain further information about the circumstances of interaction. In order to study the impact of GPR50 in TβRI-dependent signal transduction, the aim was to study the activation status of different levels from receptor phosphorylation onto gene transcription of the TGFβ signal transduction pathway in absence and presence of GPR50Δ4 and GPR50wt by the use of different techniques, as western blotting, Co-IP, nuclear extraction and reporter gene assay. Furthermore, we studied the molecular mechanisms responsible for signaling activation in the presence of GPR50.

With the purpose of putting our findings into another context, we used the gastric cancer cell line SNU638 which is deficient of an intact TβRII receptor and thus devoid of TGFβ signaling. We addressed the question, whether GPR50 has an the capacity to restore TβRI signaling activity.

For the functional assays, we decided for the MDA-MB-231 cells as a model for cancer and TGFβ signaling. MDA-MB-231 cells are epithelial mammary gland derived metastatic cells from an invasive breast cancer with highly metastatic potential. Being responsive to TGFβ findings based on utilization of this model helped to obtain knowledge about the effects of TGFβ signaling in cancer and during cancer progression. They have a hyperactivity of the Ras pathway leading to a less strong TGFβ-mediated growth inhibition as a result of the loss of myc repression (Chen et al, 2001). But still, TGFβ-dependent anti-proliferative effects are remarkable (Yang et al, 2012), thus MDA-MB-231 cells are forming an appropriate model for

studying the effects of TGFβ-mediated cytostasis. In addition, they served as a model to study the effects of metastasis formation due to their intact TGFβ signaling and it could be demonstrated, that TGFβ promotes their invasion and angiogenesis (Safina et al, 2007). These MDA-MB-231 cells also provided insight into the gene repertoire important for migration of those cells (Kang et al, 2003b) and the molecular basis for metastasis of cells had been deciphered (Padua et al, 2008). In addition, the mechanisms of bone metastasis have been dissected: homing of MDA-MB-231 cells to the bone marrow, its invasion, angiogenesis and osteolysis underlie a specific mechanism: TGFβ increases parathyroid hormone-related protein (PTHrP) secretion that leads to production of the osteolytic factor RANKL that promotes osteolysis and enables nesting of MBA-MB 231 cells in bone (Yin et al, 1999).

Though our results indicated a constitutive activation of the TβRI in the presence of GPR50, we decided to use a model with overexpression of GPR50 to induce and study the effects of a constitutive TGFβ signaling. The functional effects of GPR50 were addressed in migration studies in a wound-healing assay and proliferation effects were tested *in vitro* in an anchorage-independent-growth-assay and *in vivo* in a xenograft study.

All these different aspects should enable us, to confirm GPR50 as a new interacting partner of the TβRI, to describe its mode of action on activating TβRI in ligand- and TβRII-independent manner and to examine its functional impact on breast cancer development. The details of our project conception and results are topic of the following publication, which implicates all the principal research tasks for this thesis.

## 2. Article

*Ligand-independent activation of type I TGFß receptor (TßRI) by the orphan 7-transmembrane protein GPR50 in the absence of the TßRII*

**Stefanie Wojciech [1,2,3], Zakia Belaid-Choucair [3,4], Raise Ahmad [1,2,3], Anne-Sophie Journé [1,2,3], Avais Daulat [1,2,3], Jean-Luc Guillaume [1,2,3], Anastasia Saade [1,2,3], Nathalie Clément [1,2,3], Thomas Courivaud [5], Kenjiro Tadagaki [1,2,3], Philippe Delagrange [6], Céline Prunier [5] and Ralf Jockers[1,2,3*]**

[1] Inserm, U1016, Institut Cochin, Paris, 75014, France
[2] CNRS UMR 8104, Paris, France, 75014, France
[3] Univ. Paris Descartes, Paris, 75014, France
[4] Hôpital Necker, CNRS UMR 8147 Paris, 75015, France
[5] INSERM U938, Centre de Recherche Saint-Antoine, Paris, 75012, France
[6] Institut de Recherches SERVIER, Suresnes, 92150, France

* Correspondence should be addressed to:
Ralf Jockers, Institut Cochin, 22 rue Méchain, 75014 Paris.
Phone: +331 40 51 64 34; Fax: +331 40 51 64 30; e-mail: ralf.jockers@inserm.fr

**Short title:** Constitutive activation of TßRI by GPR50

**ABSTRACT**

The current dogma predicts that transforming growth factor-ß (TGFß) signaling is induced by TGFß binding to the type II TGFß receptor (TßRII), recruitment of TßRI into the complex and trans-phosphorylation of the GS domain of TßRI by TßRII. Here we report the formation of a molecular complex between TßRI and the orphan GPR50 receptor. Binding occurs through the respective transmembrane domains and the ATSHP motif in the cytoplasmic domain of GPR50 that competes with the ATGHP motif of FKBP12, a negative regulator of TGFß signaling, for binding to TßRI. This new complex leads to the spontaneous, ligand-independent activation of TßRI that does not require TßRII. Overexpression of GPR50 in MDA-MB-231 cells promotes *in vitro* cell migration and inhibits tumor formation in a xenograft model. Our results describe a previously unappreciated spontaneous activation mode of TßRI and identify GPR50 as a TßRI co-receptor with potential impact on breast cancer development.

## HIGHLIGHTS

1. The interaction of GPR50 and TβRI induces constitutive TβRI-dependent signaling
2. GPR50 competes with FKBP12 for binding to the TβRI
3. GPR50 activates the TβRI in a ligand- and TβRII-independent manner
4. Overexpression of GPR50 induces TGFβ-like responses in breast cancer cells

## INTRODUCTION

Transforming growth factor β (TGFß) is a cytokine, which regulates many cellular processes and plays an important role during normal embryogenesis due to its multiple effects on proliferation, differentiation, apoptosis and migration (Massague, 2012; Shi & Massagué, 2003b). The deregulation of components of the TGFß pathway is at the basis of many diseases including cancer (Ikushima & Miyazono, 2010; Massague, 2008). TGFß elicits its effects through two single-transmembrane spanning serine/threonine (Ser/Thr) kinases called type I and type II TGFß receptors (TßRI and TßRII, respectively) (Franzen et al, 1993). Binding of TGFß to TßRII triggers the recruitment of TßRI (Wrana et al, 1994). The constitutively active TßRII kinase activates TßRI by phosphorylating several Ser/Thr residues in the highly conserved GS region ($^{185}$TTSGSGSG$^{192}$) located N-terminal to the kinase domain of TßRI (Wieser et al, 1995). This induces the so-called "inhibitor-to-substrate" activatory switch, which consists in the dissociation of the FKBP12 inhibitor and the subsequent binding of SMAD2/3 proteins (Huse et al, 2001). Phosphorylation of SMAD2/3 by the TßRI kinase (Zhang et al, 1996) induces their dissociation from the receptor, which then dimerize, form a complex with the Co-SMAD, SMAD4, translocate to the nucleus, and regulate gene transcription upon DNA binding (Heldin & Moustakas, 2012). Alternatively, TGFβ can also signal through Smad-independent pathways like the activation of MAP kinases p38 and JNK (Zhang, 2009). The framework of this relatively simple pathway, which is based on one single signaling mediator that shuttles from the receptor to the nucleus to regulate gene expression, was revealed more than a decade ago (Massagué, 2000). Over the last couple of years multiple regulators have been identified that allow a context-dependent integration of the core signaling pathway (Massague, 2012). Most of them are facilitating the onset of signaling after stimulation or are regulating signaling sustainability, which underlines the primary importance of precisely controlling the TGFß/SMAD pathway (Kang et al, 2009; Xu

et al, 2012) Among these are positive regulators, such as SARA, that assists in SMAD recruitment to the TβRI (Tsukazaki et al, 1998), the inhibitory SMAD7 (Nakao et al, 1997a), which recruits E3 ligases that induce proteasomal degradation of the receptor complex (Kavsak et al, 2000) and TMEPAI, which interferes with SMAD2/3 phosphorylation (Watanabe et al, 2010).

Only little information exists about the regulation of TGFβ signaling in the absence of ligand. As eluded above, FKBP12 has been described as a gatekeeper to prevent ligand-independent signaling by locking TβRI in its inactive conformation (Chen et al, 1997; Wang et al, 1996) (Wang & Donahoe, 2004). Knowing the far-reaching consequences of TGFß signaling, tight regulation of its ligand-independent activity appears to be a crucial issue.

G protein-coupled receptors (GPCRs), also called 7-transmembrane (7TM) spanning proteins, represent the most abundant class of cell surface receptors with approximately 800 members. GPCRs are major drug targets accounting for up to 30% of currently marketed drugs (Rask-Andersen et al, 2011). Many reports indicate that GPCRs have the potential to interact with themselves (homomers) and with other GPCRs or receptors from other families (heteromers) (Maurice et al, 2011a). Within these heteromeric complexes, allosteric regulation of one protomer by the other is often observed. Among the different GPCR members, approximately 100 are considered as orphans for which no endogenous ligand has been identified so far. Apart from the ongoing deorphanization of these receptors, there is increasing evidence for ligand-independent functions of orphan GPCRs (Levoye et al, 2006c). Indeed, by physically interacting with other membrane receptors with know ligand and function, these orphan receptors can allosterically regulate the function of the latter. Complexes between GPCRs and TßR have not been described yet.

Among these orphan GPCRs figures GPR50, which shares highest sequence homology with melatonin receptors, but which lost its ability to bind melatonin during evolution (Dufourny et

al, 2008; Jockers et al, 2008). The carboxyl terminal tail (C-tail) of GPR50 of approximately 320 amino acids is one of the characteristic features of GPR50. This C-tail has been shown to scaffold several interacting partners (Grünewald et al, 2009; Li et al, 2011) and to modulate in a ligand-independent manner the activity of other membrane receptors such as the melatonin $MT_1$ receptor within heteromeric complexes (Levoye et al, 2006b). In addition, a frequent sequence variant (MAF=0.4) lacking 4 amino acids ($^{502}$TTGH$^{505}$) of the C-tail (GPR50Δ4) exists in humans and has been associated with mental disorders (Thomson et al, 2005) and altered lipid metabolism (Bhattacharyya et al, 2006).

We report here for the first time the complex formation between TßRI and GPR50, an orphan 7-transmembrane spanning GPCR. Within this complex composed of TßRI and GPR50, but not TßRII, GPR50 enhances the basal, TGFß-independent, capacity of TßRI to activate SMAD2/3, most likely by excluding the inhibitory FKBP12 from binding to TßRI and by stabilizing activatory conformations in TßRI.

## RESULTS

### GPR50 interacts with TßRI

To identify novel interacting proteins of GPR50, we applied the tandem affinity purification TAP) protocol recently optimized for GPCRs (Daulat et al, 2007) by fusing the TAP-tag to the carboxyl terminal tail of the full-length human GPR50Δ4 variant and by stably expressing the GPR50Δ4-TAP construct in HEK293T cells. Mass spectrometric analysis of purified proteins identified 5 unique peptides corresponding to the TßRI in 3 independent purifications but not in control purifications with naïve HEK293T cells (Figure 1A). Co-immunoprecipitation experiments in HEK293T cells confirmed that the human TßRI constitutively interacts with the human GPR50Δ4 variant and the GPR50wt isoform (Figure 1B). Similar results were observed in breast cancer MDA-MB-231 cells stably expressing

either GPR50Δ4 or GPR50wt (Figure 1C). To evaluate the role of TGFß activation on this interaction, HEK293T cells were stimulated for 2 hours with TGFß or transfected with the constitutively active TβRI T204D mutant (TβRI ca) (Figure 1B). The amount of co-precipitated GPR50 was not altered indicating that the TßRI/GPR50 interaction occurs independently of the activation state of TßRI. To further validate these results in intact cells, we performed bioluminescence resonance energy transfer (BRET) donor saturation experiments. The *Renilla* luciferase 8 (Rluc8) energy donor and the yellow fluorescent protein (YFP) energy acceptor were fused to the carboxyl terminus of TßRI. Co-transfection of a fixed amount of TßRI-Rluc8 expression plasmid and increasing amounts of TßRI-YFP, GPR50wt-YFP or GPR50Δ4-YFP (Figure 1D) resulted in a hyperbolic saturation curve with increasing YFP/Rluc ratios for all receptor combinations, reflecting a specific interaction between BRET donor and acceptor pairs ($BRET_{50}$=1.342±0.185, 0.025±0.006 and 0.031±0.0.010 (n=3-4)) for TßRI homomers and TßRI/GPR50wt and TßRI/GPR50Δ4 heteromers, respectively. Incubation with TGFß had no apparent effect on the BRET signal of TßRI/GPR50wt and TßRI/GPR50Δ4 heteromers within the first 30 minutes after addition (Supplementary Figure 1A,B). Expression of TßRI-Rluc8 donor with the insulin receptor (IR)-YFP or leptin receptor (OBRa)-YFP negative controls resulted in a linear, non-saturable BRET increase, characteristic of random interactions. Overall, co-immunoprecipitation and BRET experiments confirm the formation of a constitutive TßRI/GPR50 complex in HEK293T and MDA-MB-231 cells.

Formation of a TßRI/TßRII complex is an obligatory step of the current TGFß receptor activation model. To verify whether TßRII is necessary for the formation of the TßRI/GPR50

complex, we used gastric carcinoma SNU 638 cells, which are devoid of TβRII.

Figure 1

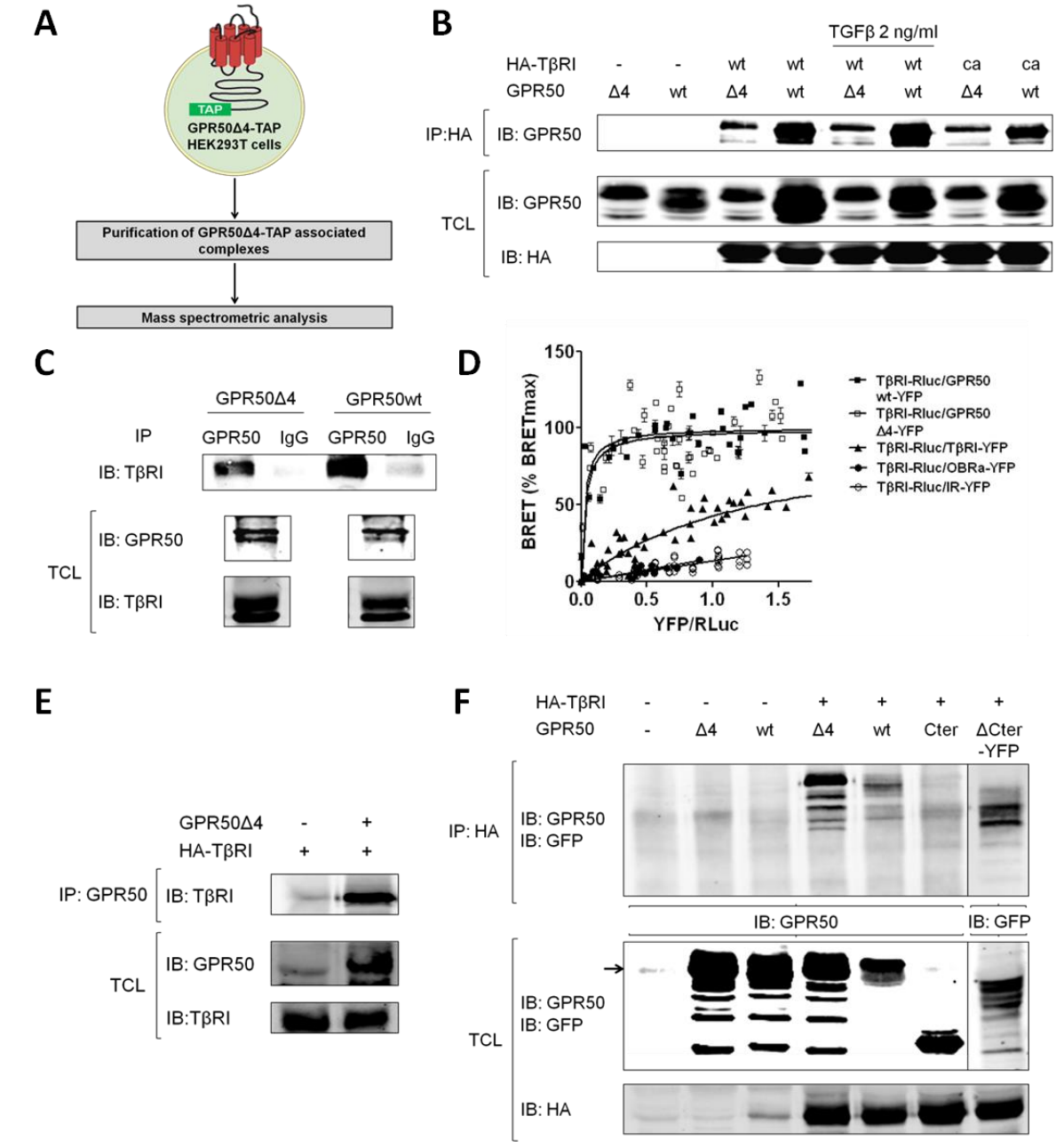



**Figure 1. GPR50 interacts with the TβRI**

(A) Tandem affinity purification was performed with naïve HEK293T cells or HEK293T cells stably expressing GPR50Δ4-TAP. After purification, mass spectrometry was employed for protein identification.

(B) HEK293T cells were co-transfected with GPR50Δ4 or GPR50wt and HA-TβRI-wt or HA-TβRI-ca. Stimulation with 2 ng/ml TGFβ was done for 1 h. Cell lysates were subjected to co-immunoprecipiation with anti-HA antibody and blotted against GPR50. Expression was checked in total lysates using anti-GPR50 and anti-HA antibodies.

(C) MDA-MB-231 stably expressing GPR50Δ4 or GPR50wt cell lysates were precipitated with an anti-GPR50 antibody, the binding of TβRI was checked with an anti-TβRI antibody. Lysates were used to reveal the total expression level.

(D) For BRET saturation curves, HEK293T were transfected with a constant amount of TβRI-Luc and increasing doses of TβRI-YFP, GPR50Δ4-YFP or GPR50wt-YFP. IR-YFP and OBRa-YFP served as negative control. BRET signals were measured after coelenterazine addition.
(E) SNU638 cells were co-transfected with HA-TβRI and GPR50Δ4 plasmids, GPR50 was precipitated with an anti-GPR50 antibody and blotting against TβRI was performed with an anti-TβRI antibody. Total lysates were used as expression control.
(F) HEK293T cells were transfected with HA-TβRI and the indicated GPR50 constructs. Lysates were precipitated with an anti-HA antibody and blotted against GPR50 and GFP. Total lysates were analyzed for expression with anti-HA-, anti-GPR50- or anti-GFP antibody.

TßRI was readily co-immunoprecipitated with GPR50Δ4 in these cells indicating that TßRII is not necessary for the formation of the TßRI/GPR50 complex (Figure 1E).

To characterize the molecular determinants involved in the interaction, we expressed the TM domain (GPR50ΔCter) and the carboxyl terminus of GPR50 (GPR50Cter) separately (Figure F). Only the GPR50ΔCter construct co-immunoprecipitated with TßRI indicating that the TM region of GPR50 contains the major molecular determinants necessary for the TßRI/GPR50 interaction. Taken together, the TßRI/GPR50 complex is insensitive to TGFß stimulation, occurs in the absence of TßRII and involves predominantly the TM domain of GPR50.

**Expression of GPR50 induces basal TβRI-dependent signaling**

Our next aim was to study the effects of GPR50 expression on TGFβ signaling. We first evaluated the basal SMAD2/3 phosphorylation state, which was dramatically increased in the presence of GPR50 (Figure 2A,B). The effect was prevented by pre-incubating cells with the TßRI kinase specific SB 431542 inhibitor (Figure 2C). Increased basal activation of the SMAD pathway in the presence of GPR50 was also observed at further downstream steps like complex formation between SMAD2/3 and SMAD4 (Figure 2D,E), nuclear translocation of SMAD2/3 (Figure 2F) and ARE- and CAGA-dependent reporter gene assays (Figure 2G,H). Collectively, the GPR50Δ4 variant showed more pronounced effects on basal activity of the SMAD pathway than the GPR50wt variant. Depending on the specific signaling event

monitored, TGFß stimulation had only marginal to significant effects on the increased basal activation of the SMAD pathway in the presence of GPR50 than the GPR50wt variant.

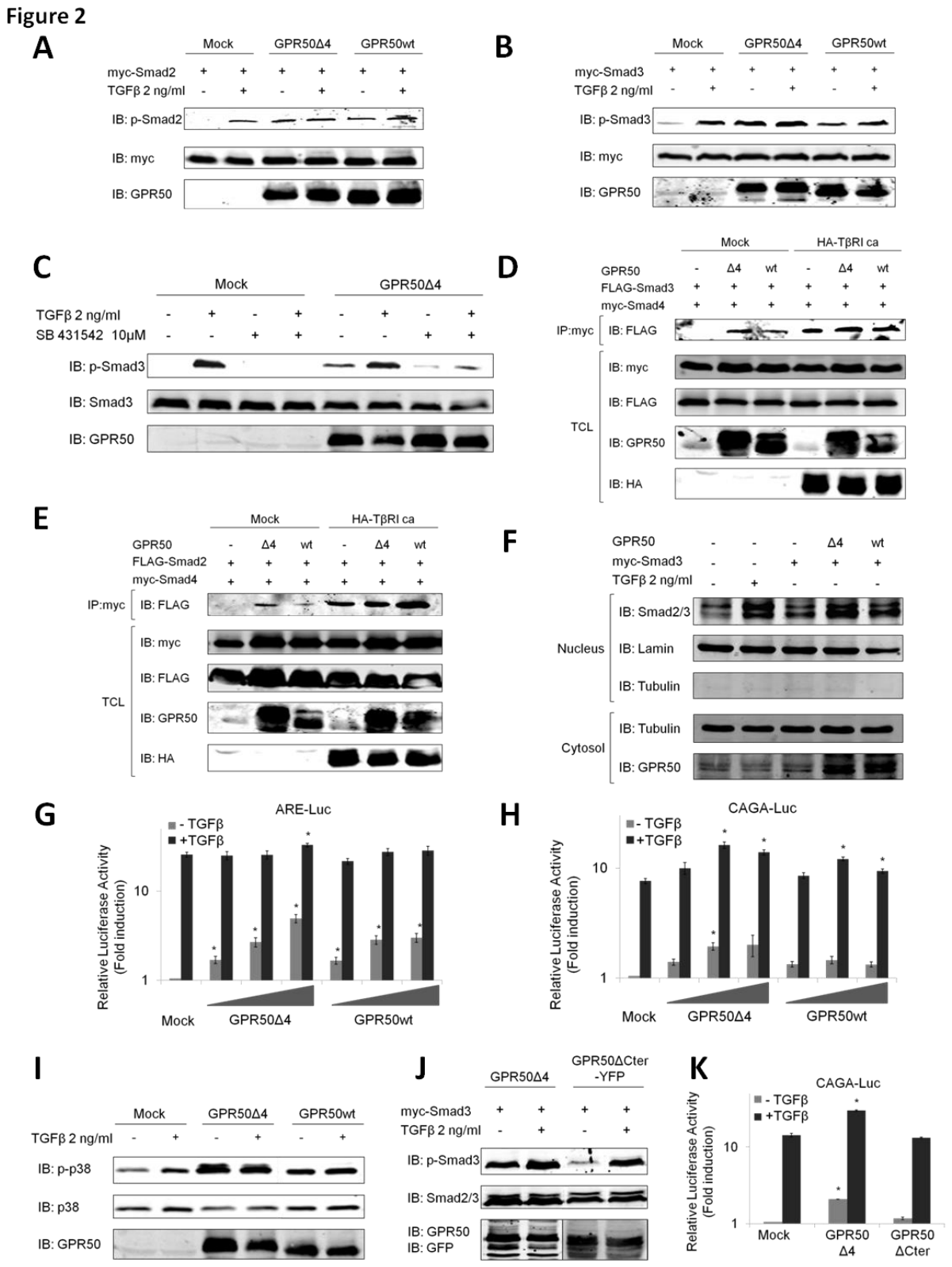



**Figure 2. Expression of GPR50 induces ligand-independent activation of TβRI-dependent signaling**

(A and B) HEK293T cells were transfected with myc-SMAD2 (A) or myc-SMAD3 (B), and GPR50Δ4 or GPR50wt. Cells were starved overnight and stimulated with 2 ng/ml TGFβ for one hour. To test SMAD2 phosphorylation, myc-SMAD2 was enriched with preceding precipitation with an anti-myc antibody. SMAD3 lysats were directly separated by SDS-PAGE. Immunoblots were revealed with p-SMAD2- or p-SMAD3 antibodies respectively, anti-SMAD2/3 and anti-GPR50 antibodies were used to check expression.
(C) HEK293T cells were transfected and prepared as described in (A and B) but additionally treated overnight with 10 µM of SB 431542 TβRI-kinase activity inhibitor before stimulation.
(D and E) To check SMAD2- or SMAD3 complex formation with SMAD4, cells were transfected with corresponding myc- or FLAG-tagged constructs, respectively, in the absence or presence of GPR50Δ4 and GPR50wt. Additional transfection of a constitutive active HA-TβRI-ca served as positive control. SMAD4 was precipitated with an anti-FLAG antibody and bound SMAD2 or SMAD3 was revealed upon blotting against SMAD2 or SMAD3 via the myc-tag. Total lysates were used to determine total expression.
(F) Cells were transfected as in (A and B) and nuclear extracts were prepared. The presence of SMAD3 in the nucleus was detected by the use of an anti SMAD2/3 antibody. Lamin- and Tubulin-expression were checked to ensure purity of the nuclear extracts and GPR50 expression was controlled in the cytosolic fraction.
(G and H) To test TGFβ-dependent transcription of genes, HeLa cells were transfected with a *Firefly*-Luciferase coupled ARE- or CAGA- promoter construct and a *Renilla* Luciferase for normalisation. Increasing doses of GPR50Δ4 and GPR50wt were added to the cells. Cells were stimulated overnight with 2 ng/ml of TGFβ and luciferase activity was measured. Figures show a representative experiment performed in triplicates as mean ± SEM ( * = p<0,05).
(I) Cell were transfected as in (A and B), cell lysates were checked for phosphorylation of p38 with a phospho-p38 antibody. Total p38 and GPR50 plasmid expression were blotted as control.
(J) HEK293T cells were transfected with GPR50Δ4 and GPR50ΔCter and treated as described in (A and B). GPR50 expression was revealed with an anti-GPR50 or -GFP antibody.
(K) For reporter gene assay, HeLa cells were transfected with the CAGA-*Firefly* Luciferase construct, the *Renilla* Luciferase and constant doses of GPR50Δ4 and GPR50ΔCter. Cells were further treated and analyzed in reporter gene assay as in (G and H).

Depending on the specific signaling event monitored, TGFß stimulation had only marginal to significant effects on the increased basal activation of the SMAD pathway in the presence of GPR50. Significantly increased basal activation was also observed for the non-canonical p38 signaling pathway (Hanafusa et al, 1999; Yu et al, 2002) in the presence of GPR50Δ4 with no further increase upon TGFß treatment and to a much smaller extend for GPR50wt suggesting that TGFß-independent activation of TßRI by GPR50 is not restricted to the SMAD2/3 pathway but can also be extended towards the p38 pathway (Figure 2I).

We next wanted to define the molecular determinants of GPR50 involved in increased TGFβ signal transduction. Surprisingly, the GPR50ΔCter construct was unable to increase the basal SMAD phosphorylation suggesting that despite the fact that the TM domain of GPR50 is involved in the interaction with TßRI, it is not sufficient for the functional effect on the SMAD pathway (Figure 2J). The absence of effect of the GPR50ΔCter construct was further

confirmed in the CAGA reporter gene assay (Figure 2K). These observations suggest that the cytosolic C-tail of GPR50 is somehow involved in the functional effect of GPR50 on the SMAD pathway, a hypothesis that is also consistent with the differential effects observed for the two GPR50 variants that differ by a 4 amino acid deletion/insertion in the C-tail.

Collectively, these data show that GPR50 promotes TßRI-dependent signaling in the absence of TGFβ ligand through the SMAD2/3 and p38 pathways. This effect tends to be more pronounced for the GPR50Δ4 variant and relies on the C-tail of GPR50 and the TßRI kinase activity.

**GPR50 interferes with FKBP12 binding to TßRI**

We then set out to identify the molecular mechanism by which GPR50 potentiates the basal activation of the TßR/SMAD pathway. Initial radioligand binding competition experiments with $^{125}$I-TGFß could rule out the potential modification of TGFß receptor cell surface expression or improved affinity of TGFß for its receptor (Supplementary Figure 2A). Furthermore, induction of TGFß production or secretion of cells by GPR50 expression is unlikely to occur as supernatants of cells expressing GPR50 were unable to promote SMAD phosphorylation in naïve cells (Supplementary Figure 2B).

The absence of effect of the GPR50ΔCter construct on the basal activation of the SMAD pathway hints to the potential implication of the C-tail of GPR50 in this effect. Along this line, we decided to investigate the potential effect of GPR50 on proteins interacting with the TßR. We first verified the possibility of a direct interaction of GPR50 with SMAD2 or SMAD4 that might facilitate the recruitment of these proteins to the TßR and thus potentiate the response of the TßR/SMAD pathway. Co-immunoprecipitation experiments between GPR50 and SMAD2 or SMAD4 did not provide any support for this hypothesis (Supplementary Figure 3). We then focused our attention on FKBP12, which binds to TßRI in

its inactive conformation thus stabilizing TßRI in its inactive state and preventing SMAD2/3 binding (Chen et al, 1997; Huse et al, 2001). FKBP12 has been classified as TGFß signaling gatekeeper as its depletion is causing hypersensitivity towards TβRII, leading to transphosphorylation of TβRI and subsequent induction of downstream signaling. We therefore tested the influence of GPR50 on the interaction of TßRI and FKBP12 by coimmunoprecipitation. Coexpression of GPR50Δ4 and GPR50wt variants diminished the amount of FKBP12 interacting with TßRI by 70% and 40%, respectively (Figure 3A). This effect could not be explained by the scavenging of FKBP12 by GPR50, as GPR50 did not interact directly with FKBP12 (Figure 3B). Collectively, these results suggest that GPR50 competes with FKBP12 for binding to TßRI.

To further characterize the relationship between FKBP12 and GPR50, we used the FK506 macrolide compound, which is known to bind to FKBP12 at a site that overlaps with binding to TßRI (Wang et al, 1994). Pre-incubation of cells with FK506 in the absence of GPR50 triggered indeed the expected increase in basal SMAD3 phosphorylation, an effect that was not further increased in the presence of GPR50 (Figure 3C). The overexpression of FKBP12 completely abolished basal SMAD3 phosphorylation irrespective of the absence of presence of GPR50. The fact that the effects of GPR50 on basal SMAD3 phosphorylation can be blocked by FKBP12 overexpression and that the effects of GPR50 and FK506 are not additive are compatible with a common action mechanism, the competition of GPR50 and FK506 with FKBP12 for TßRI binding. To further verify this hypothesis, we aimed to identify the molecular basis of this competition. Sequence analysis of the C-tail of GPR50 and FKBP12 revealed the existence of a repetitive 5 amino acid motif (**A**XZ**HP**) (X=A, T, S; Z=G, S) in GPR50 that is similar to the $^{84}$ATGHP$^{88}$ motif of FKBP12 (Figure 3D, left part). Interestingly, the $^{84}$ATGHP$^{88}$ motif of

FKBP12 corresponds to a loop that is part of the binding pocket in the co-crystal structure of

Figure 3

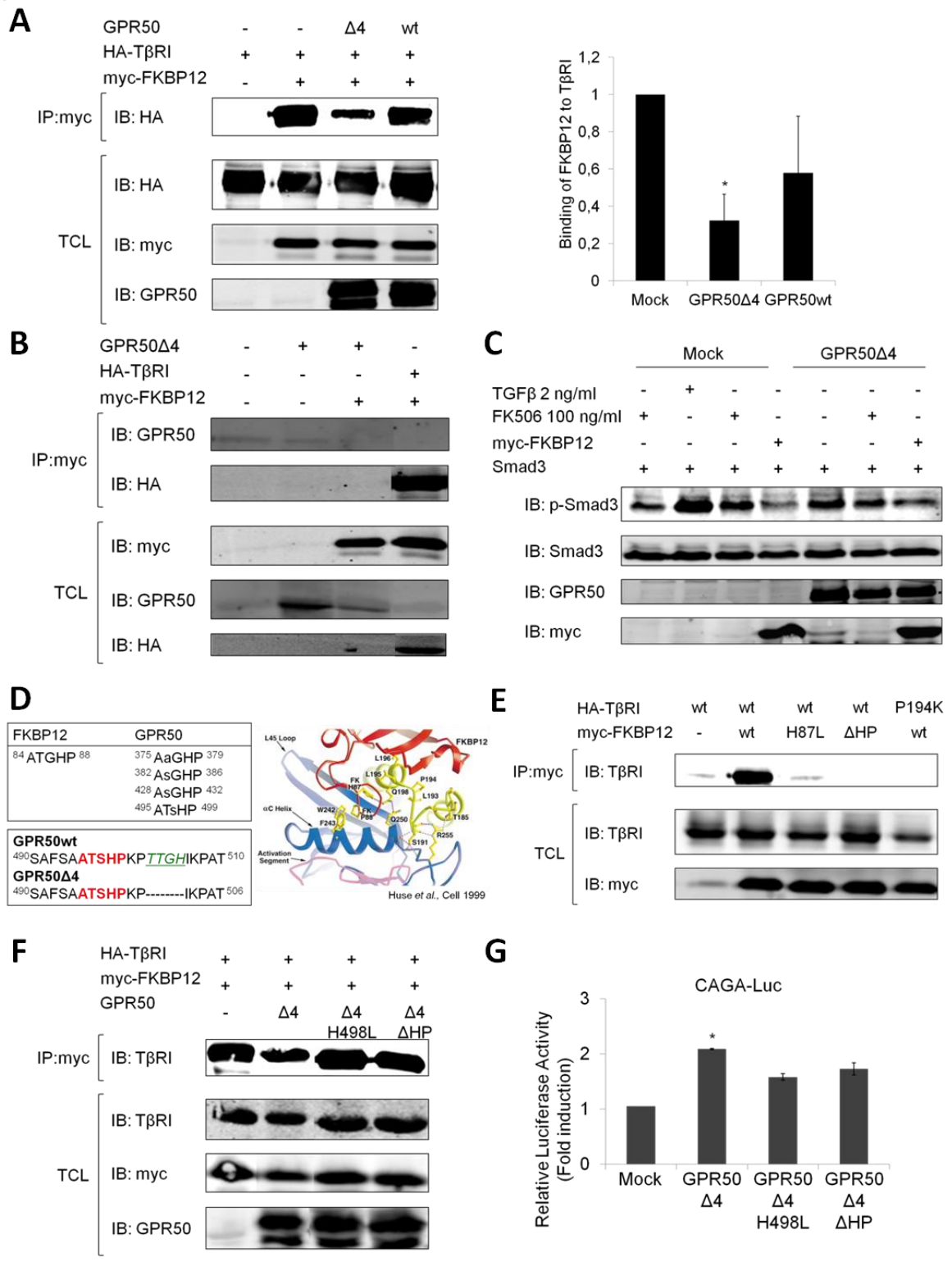



**Figure 3. GPR50 is competing with FKBP12 for the binding to TβRI due to a similarity motif**
(A) (Left) HEK293T cells were transfected with HA-TβRI and myc-FKBP12 and either GPR50Δ4 or GPR50wt. Lysats were precipitated for FKBP12 using an anti-myc antibody and blotted with an anti-TβRI to reveal complex formation. Total lysats were addressed for expression of myc-FKBP12, HA-TβRI and GPR50 with corresponding antibodies. (Right) Three independent experiments were densitometrically analyzed and data represent the mean ± SEM ( * = p<0,05).
(B) Co-immunoprecipation was performed according (A) with cells co-transfected with myc-FKBP12 and either GPR50Δ4 or HA-TβRI. Precipitates were blotted against GPR50 and TβRI.

(C) HEK293T cells were transfected with myc-SMAD3, myc-FKBP12 and GPR50Δ4 as indicated. Cells were starved and stimulated for 1h with 2 ng/ml of TGFβ or of 100 ng/ml FK506. Total lysates were immunoblotted for SMAD3-phosphorylation and total expression of myc-SMAD3, GPR50 and myc-FKBP12 with suitable antibodies.
(D) Sequence alignment of FKBP12 and GPR50 revealed sequence similarities between a C-terminal motif in FKBP12 and a repetitive sequence in GPR50 (upper left panel). Analysis of localization of the repetitive motifs in GPR50Δ4 and GPR50wt shows the proximity of one to the Δ4 deletion (lower left panel). Existing structural data from Huse et al. (Huse et al, 1999) highlight the implication of the HP loop in binding to the TβRI (right).
(E) HEK293T cells were co-transfected with HA-TβRI or the HA-TβRI-P194K mutant and the indicated FKBP12 constructs. Co-immunoprecipitation was performed as in (A).
(F) HEK293T cells were transfected with indicated plasmids and precipitated as in (A)
(G) Reportergene assay was performed as in (2 G and H) with cells transfected with constant doses of the GPR50 constructs. Figure shows one representative experiment performed in triplicates as mean ± SEM ( * = p<0,05).

FKBP12 and the unphosphorylated GS region and kinase domain of TßRI (Huse et al, 1999) (Figure 3D, right part). To directly demonstrate the importance of the ATGHP loop of FKBP12 in TßRI binding, we designed two mutants predicted to abolish FKBP12 binding to TßR1 (FKBP12-H87L, FKBP12ΔHP). Figure 3E shows that both mutants are unable to bind TßRI similar to the previously reported TßRI-P194K mutant that served as a positive control of the loss of interaction (Chen et al, 1997).

To address the importance of the **A**XZ**HP** motifs in GPR50, we concentrated on the $^{495}$ATSHP$^{499}$ motif located next to the $^{502}$TTGH$^{505}$ deletion in the GPR50Δ4 variant. Disruption of the $^{495}$ATSHP$^{499}$ motif (H498L, ΔHP) in the GPR50Δ4 variant fully restored FKBP12 binding to levels seen in the absence of GPR50 (Figure 3F). Similar observations were made at the reporter gene level using a CAGA promoter construct underlining the importance of the $^{495}$ATSHP$^{499}$ motif of GPR50 (Figure 3G).

Taken together, whereas GPR50 and TßRI interact mainly through their respective transmembrane domains, the functional effect of GPR50 on TßRI signaling relies on the $^{495}$ATSHP$^{499}$ motif in the C-tail of GPR50 that competes with the ATGHP loop of FKBP12 for binding to TßRI. Displacement of FKBP12 contributes at least partially to the potentiation of the basal activity of the TßR/SMAD pathway in the presence of GPR50. The proximity of the $^{495}$ATSHP$^{499}$ motif in GPR50 to the $^{502}$TTGH$^{505}$ deletion might be at the origin of the

more pronounced effect of the GPR50Δ4 variant as compared to GPR50wt to modulate the TßR/SMAD pathway.

**GPR50 activates TβRI-dependent signaling in the absence of TβRII**

According to the current dogma, TßRII fulfils two essential functions in the TßR activation process, namely binding of TGFß and transphosphorylation of TßRI in the GS region (Wieser et al, 1995). As our results presented in figure 1E show that formation of the GPR50/TßRI complex does not require TßRII, we asked the question whether TßRI with GPR50 could activate SMAD3 in the absence of TßRII. Native SNU638 are indeed devoid of any TGFß response as monitored at the level of SMAD3 phosphorylation and expression of TßRII in these cells restores the TGFß responsiveness (Figure 4A). Signaling through this pathway was similarly restored by exogenous TßRII expression at the level of CAGA-driven reporter gene activation (Figure 4B). A potentiating effect of GPR50 on the basal activation of the TßR/SMAD pathway in the absence of TßRII was observed on the level of SMAD3 phosphorylation in SNU638 cells (Figure 4C). This effect was entirely dependent on the TßRI kinase activity, as sensitive to the SB 431542 inhibitor (Figure 4D). A similar increase in basal activity in the presence of GPR50 was observed at the level of the CAGA reporter gene assay (Figure 4B). No further increase was observed upon TGFß addition, as expected, in the absence of TßRII. To explore the possibility that the effect of GPR50 is dependent on FKBP12 release from TβRI as observed in the presence of TßRII, we overexpressed FKBP12 in SNU638 cells and monitored SMAD3 phosphorylation. Overexpression of FKBP12 clearly reduced the effects evoked by GPR50 further supporting the hypothesis that GPR50 competes with FKBP12 for binding to TβRI (Figure 4E).

Taken together, we established that GPR50 expression in SNU638 cells can induce ligand-independent activation of TβRI/SMAD signaling, which is sensitive to FKBP12 expression

levels and dependent on TßRI kinase activity. Activation of TßRI kinase activity in the absence of TßRII establishes a new operation mode of TßRI in the complex with GPR50.

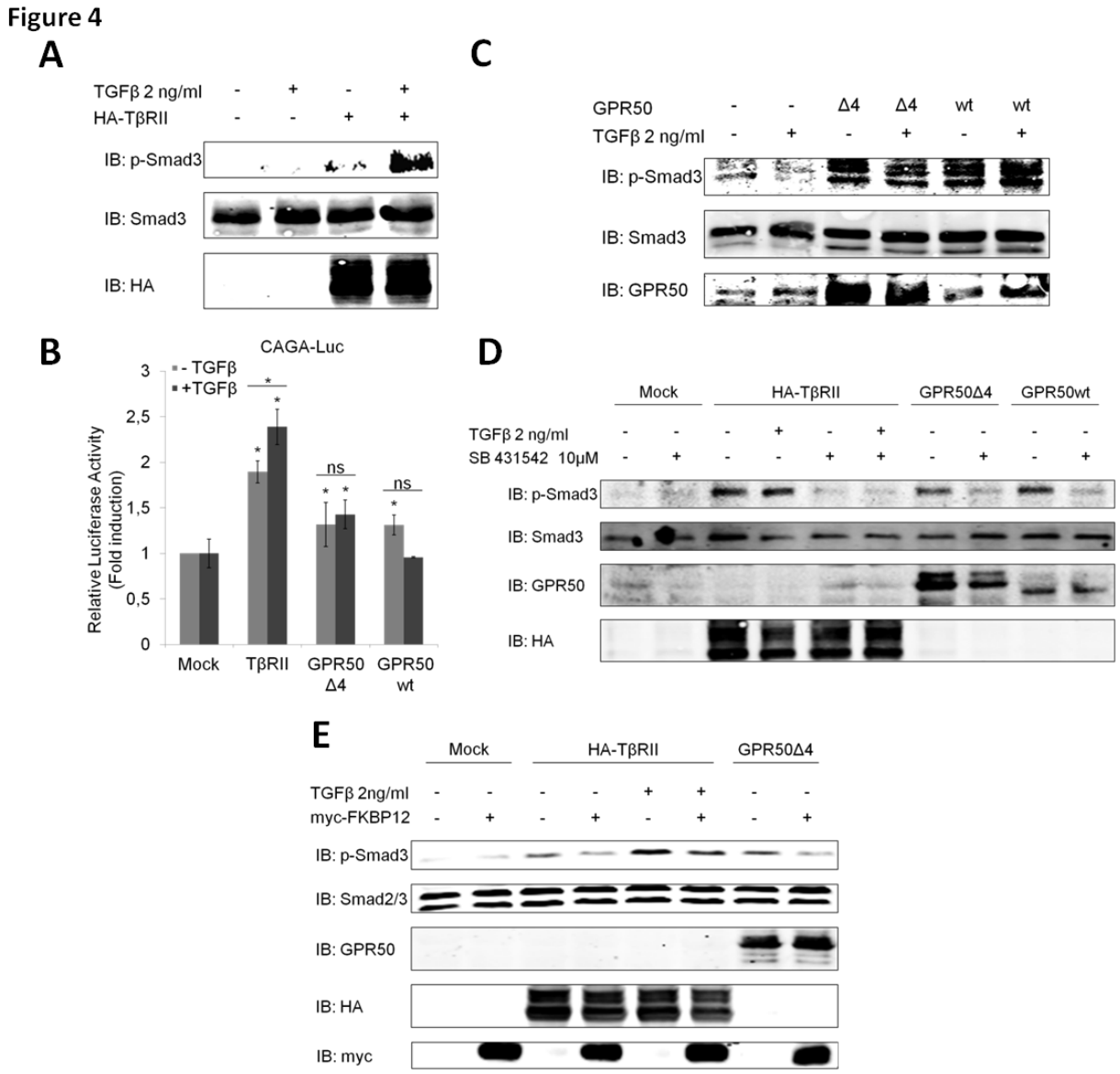



**Figure 4. SNU638 cells show the capacity of GPR50 to activate TβRI independently of TβRII.**
(A) SNU638 cells were transfected either with Mock or HA-TβRII plasmid and stimulated for one hour with 2 ng/ml of TGFβ. Lysats were immunoblotted for phospho-SMAD3 and total SMAD3 and expression of TβRII plasmid was revealed with an anti-HA-antibody.
(B and D) SNU638 cells were transfected with the indicated plasmids, stimulated during one hour with 2 ng/ml TGFβ and in (D) additionally pretreated over night with SB 431542 at 10 μM. Cells were lysed and blotted as described before in (2A and B).
(C) For the reporter gene assay HA-TβRII-, GPR50Δ4- and GPR50wt transfected SNU638 cells were treated as in (2H). Data represent the mean ± SEM (* = p<0,05) of one representative experiment performed in triplicates.
(E) SNU638 cells were transfected with the indicated plasmid and lysats were blotted with an pSMAD3 antibody. Additionally, HA-TβRII, GPR50 and FKBP12 expression were verified with corresponding antibodies.

**GPR50 expression in MDA-MB-231 cells is sufficient to promote TGFβ resembling effects**

To further establish the functional relevance of GPR50 expression on the TßR/SMAD2/3 pathway, we choose the well-described and widely used TGFβ responsive MDA-MB-231 breast cancer cell line We first recapitulated the ligand-independent basal signaling in the presence of GPR50 and the presence of the GPR50/TßRI complex in this cell model (Figure 5A) and then the interaction between GPR50 and TßRI (Figure 5B). We then established stable cell lines expressing similar levels of GPR50wt or GPR50ΔCter (Figure 5C) and performed several functional tests. The effect of GPR50 expression on the migratory ability of MDA-MB-231 cells was assessed in the wound healing assays (Figure 5D). Analysis of wound closure after 30 hours showed, that the presence of GPR50 in MDA-MB-231 cells increased the migratory capacities of these cells as seen by the higher number of cells in the wound area (Figure 5D). The same effect was observed when cells were treated during the wound healing process with TGFβ, suggesting a common mechanism (Figure 5D). In the soft agar anchorage-independent growth assay, cells stably overexpressing the GPR50Δ4 variant showed an inhibition of 60% in the number of colonies formed during nearly 3 weeks (Figure 5F). Apart from the number of colonies, their size was also reduced in cells expressing the GPR50Δ4 variant as compared to mock and GPR50wt transfected cells. Similar results were obtained in xenograft experiments. Monitoring tumor growth over 34 days in nude mice injected with MDA-MB-231 cells in the flanks revealed that the presence of GPR50wt or GPR50Δ4 slows down tumor growth compared to mock-injected mice starting from day 23 until the end (day 34) (Figure 5E).

Figure 5

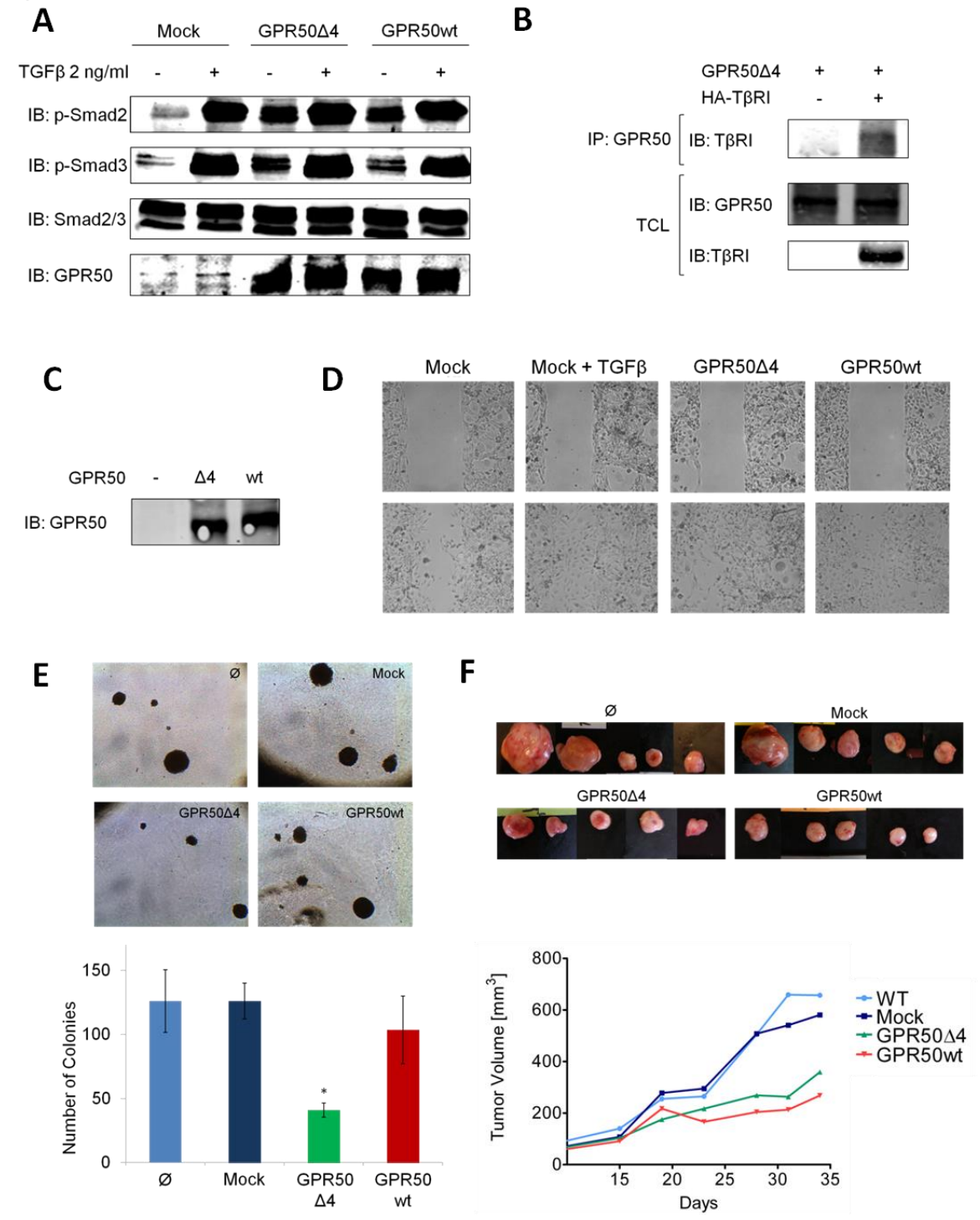



**Figure 5. Overexpression of GPR50 in MDA-MB-231 cells induces promigratory and antiproliferative effects.**

(A) MDA-MB-231 cells were transiently transfected with GPR50Δ4 or GPR50wt and lysats were tested for SMAD 2 and SMAD 3 Phosphorylation and SMAD 2/3 and GPR50 total expression.

(B) For the Co-IP, MDA-MB-231 cells were transiently transfected as indicated and IP was performed as indicated in (1E).

(C) Analysis of GPR50 expression in lysates of stably overexpressing MDA-MB-231 cells

(D)MDA-MB-231 cells were seeded into IBIDI µ plates, after removal of the insert, cells were stimulated with 2 ng/ml of TGFβ and migration of the cells and surface closure was monitored every 6 hrs. Images show representative migration after 30 hrs of one experiment out of three.

(E) Anchorage-independent growth assay of MDA-MB-231 cells stably overexpressing GPR50 monitored for 18 days. Images show an example of colony size and distribution. Diagram shows the mean value ± SEM of the colony number of 3 dishes for each condition in one representative experiment.
(F) Xenograft experiments after injection of MDA-MB-231 cells into the flanks of nude mice. Images show 5 representative out of 10 (8 for the GPR50Δ4 condition) tumors. The graph shows the development of the tumor growth during 34 days.

## DISCUSSION

We describe here a previously unappreciated activation mode of TßRI when engaged into a molecular complex with the orphan GPR50 receptor. Whereas in the classical mode of action binding of TGFß to TßRII promotes the association and phosphorylation of TßRI by TßRII, complex formation of TßRI with GPR50 leads to a spontaneous and ligand-independent activation of TßRI and induction of downstream signaling through TßRI regulated pathways. Spontaneous activation of TßRI involves reduced binding of FKBP12 to TßRI and was sufficient to promote several TGFß-like physiological responses like promotion of migration, reduction of anchorage-independent growth *in vitro* and reduction in tumor growth in a xenograft mouse model. Complex formation between GPR50 and TßRI is likely to fine-tune the TßRI signaling capacity in a cell context-dependent manner, defines a new ligand-independent function for the orphan GPR50 receptor and represents the first example of direct crosstalk between a member of the GPCR super-family and TßRs.

### Activation mechanism of TßRI in the GPR50/TßRI complex

Our results indicate that TßRI activation in the GPR50/TßRI complex is different from the activation mode in the TßRII/TßRI complex. Whereas TßRI activation in the TßRII/TßRI complex is dependent on TGFß binding to TßRII, complex formation with TßRII and phosphorylation of TßRI by TßRII (Wrana et al, 1994), the GPR50/TßRI complex appears to be independent of TßRII interaction and phosphorylation. In contrast, both activation modes

Figure 6

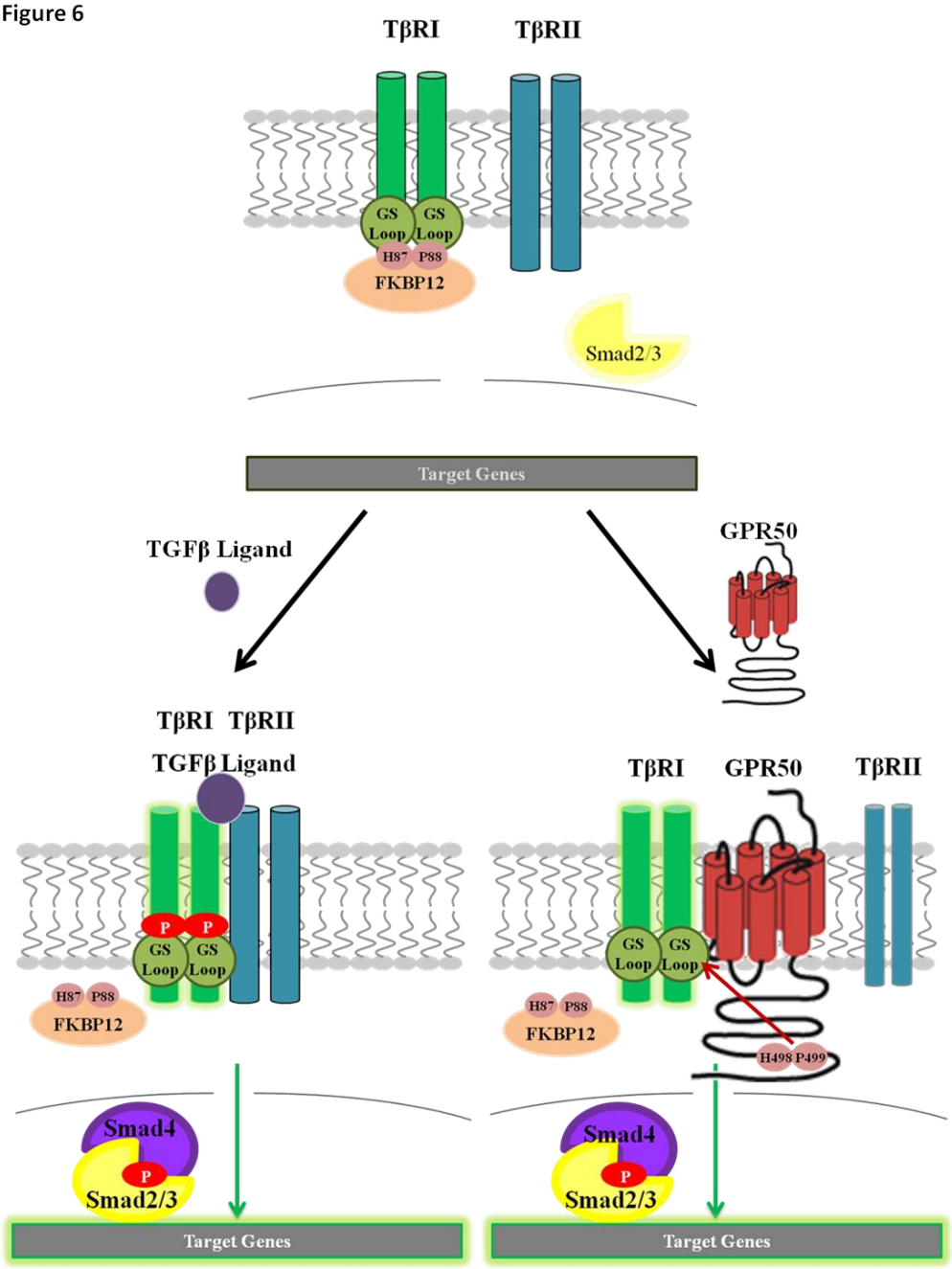



**Figure 6. Proposed Model of GPR50 action on TβRI and TβRI-dependent signaling**
In the basal state (upper part), the TβRI and the TβRII form homodimers, that are apart from each other. The TβRI is stabilized in its inhibitory conformation by FKBP12 where H87 and P88 contact the region around the GS domain. The R-SMADs are non-phosphorylated in the cytosol and no transcription of target genes occurs. In the classical activation mode (lower left side), the TGFβ ligand binds to the TβRII, which enables recruitment of TβRI into the complex. The TβRI gets phosphorylated in the GS domain by the TβRII, what is accompanied by a dissociation of FKBP12 and the stabilization of the active conformation. Hence, the TβRI exerts its kinase activity on the R-SMADS 2/3, which in turn form a complex with Smad4, translocate into the nucleus, bind to DNA and regulate gene transcription. If the TβRI forms a complex with GPR50 (lower right side), GPR50 induces the dissociation of FKBP12 from the TβRI due to a similarity motif of the amino acids H87 and P88 in its C-tail on position 498 and 499. Furthermore, GPR50 stabilizes the active conformation of the TβRI, that can signal downstream even in the absence of TβRII-mediated phosphorylation, in a classical manner via SMAD2/3 and SMAD 4 to the nucleus where they regulate gene expression.

converge further downstream on the level of TßRI kinase activation, which is necessary for SMAD2/3 signaling. These results raise the question how the TßRI kinase is activated in the TßRI/GPR50 complex in the absence of TßRII? The simplest explanation would be that GPR50 behaves as a TßRII-like receptor that contains a kinase that phosphorylates TßRI. Since the cytoplasmatic domain of GPR50 shows no homology to any know kinase, this scenario appears very unlikely. A further option might be that GPR50 recruits a kinase into the TßRI/GPR50 complex, which then phosphorylates TßRI. Although we are unable to rule this possibility completely out at the current stage, no Ser/Thr kinase associated with GPR50 has been identified in our TAP assay and there are no such candidates reported in the literature. To better understand the potential influence of GPR50 on TßRI, a closer look on the functional consequences of TßRI phosphorylation might be insightful. Phosphorylation of TßRI by TßRII on a yet to be identified site is believed to dissociate FKBP12 from TßRI thus liberating the two contact points of FKBP12 on TßRI, the L45 loop and the GS region, the latter, which can then be phosphorylated by the TßRII kinase at multiple Ser/Thr residues in the GS sequence (TTSGSGSG). A further, more indirect, consequence of FKBP12 dissociation is the destruction of the inhibitory wedge that inhibits the TßRI kinase in the presence of FKBP12. In the next step, SMAD2/3 proteins are recruited to TßRI through the phosphorylated GS sequence and the L45 loop of TßRI completing the so-called “inhibitor-to substrate-binding switch” (Huse et al, 2001). SMAD2/3 signaling is then initiated by phosphorylating SMAD2/3 by TßRI and dissociation of P-SMAD2/3.

What happens in the GPR50/TßRI complex? We have shown that the $^{495}$ATSHP$^{499}$ motif of GPR50 competes with the $^{84}$ATGHP$^{88}$ motif of FKBP12 for binding to the L45 loop of TßRI. Apart from this similarity, the binding mode of FKBP12 and GPR50 to TßRI appears to be different. Formation of the inhibitory wedge is unlikely to occur in the GPR50/TßRI complex as TßRI is constitutively active in this complex. Binding of GPR50 to the GS region, if it

occurs at all, will be different from binding of FKBP12 as the latter is highly dependent on the activation state of TßRI and the phosphorylation status of the GS sequence. Indeed, GPR50 binding is independent of the activation state of TßRI. Altogether, this shows that GPR50 competes with FKBP12 for binding to TßRI but the binding modes are likely to be fundamentally different providing a plausible molecular bases for the striking functional differences of FKBP12 binding (inhibition of TßRI) and GPR50 binding (activation of TßRI). Several questions remain still open concerning the precise impact of GPR50 on TßRI activity. For example, is the absence of FKBP12 in the GPR50/TßRI complex sufficient to explain constitutive phenotype? The amplitude of the SMAD2/3 signaling pathway activation of the GPR50/TßRI complex and independence of TßRII, together with the likely differences in the interaction modes of FKBP12 and GPR50 with TßRI argue for a possible additional effect on the stabilization of active TßRI conformation by GPR50. A second open question concerns the phosphorylation status of the GS sequence in the GPR50/TßRI complex. Based on the absence of the TßRII kinase, the GS sequence is predicted to be unphosphorylated. Furthermore, according to our current knowledge, TßRI is unable to phosphorylate itself yet its kinase activity is required for signaling (Bassing et al, 1994; Carcamo et al, 1995). Whether this is also true in the GPR50/TßRI complex remains to be established. Lastly, if the GS sequence turns out to be unphosphorylated, the binding mode of SMAD2/3 to the GPR50/TßRI complex is likely to be different. An intrinsic affinity of SMAD2/3 for GPR50, suggesting the stabilization of a common complex, can be ruled out according to our data.

**Other constitutively active forms of TßRI:**

Constitutive TßRI activity has been previously observed for receptors of the TGFß family. The TßRI-T204D mutant, which does not interact with TßRII anymore, constitutively activates the SMAD2/3 signaling pathway. Position 204 is part of the RTI sequence adjacent

to the kinase domain, which is not phosphorylated itself but has a positive allosteric effect on the phosphorylation of the GS sequence. This mutant receptor does not interact anymore with FKBP12 and shows increased TßRI kinase activity *in vitro* (Wieser et al, 1995). This mutant shows that, similar to GRP50, activation of TßRI is possible in the absence of TßRII, most likely by stabilizing an active conformation of TßRI. However, in contrast to the GPR50/TßRI complex, the TßRI-T204D mutant is still sensitive to TßRII as TGFß stimulation generates a further increase of SMAD2/3 signaling.

Another reported case of a constitutively active TßRI is the naturally occurring R206H mutant of the activin A receptor type I (ACVRI). This mutant is associated with fibrodysplasia ossificans progressiva, a rare genetic and catastrophic disorder characterized by progressive heterotopic ossification (Song et al, 2010). Similar to the constitutively active T204D mutation of TßRI, the R206H mutation is located in the part of the GS region that is close to the kinase domain and that allosterically regulates phosphorylation of the GS sequence. Molecular analysis revealed indeed a modest constitutive activity and impaired FKBP12 binding of the R206H mutant (Groppe et al, 2011). Recent studies indicate that the simple presence of TßRII, but not its kinase activity nor TGFß binding capacity, is necessary for the constitutive activity of the R206H mutant. This suggests that in the context of an activating TßRI mutant, the scaffolding function of a co-receptor like TßRII is sufficient for TßRI signaling (Bagarova et al, 2013).

More evidence for constitutive TßRII-independent activation of TßRI comes from DAF-1, the TßRI of *C. elegans* (Gunther et al, 2000). Interestingly, signaling of DAF-1 can occur in the absence of TßRII (DAF-4) kinase activity and promote larval development. Differences in the structure of the GS region of DAF-1 in comparison to other TßRI isoforms are possibly at the origin of this autonomous signaling capacity of TßRI. In addition, DAF-1 can also signal through the more classical DAF-1/DAF-4 complex. This example suggests that the TßRII-

independent signaling mode might have occurred early in evolution providing different options to fine-tune the TßRI signaling capacity.

Furthermore, the recently reported activation of TßRI by exposing glomerular mesangial cells to stretch in the absence of any TGFß provides further supports for the existence of alternative activation modes of TßRI (Chen et al, 2013)

Taken together these examples support the notion that TßRI has an intrinsic capacity to be constitutively active and that this activity can be assisted/amplified by the presence of other receptors like TßRII or GPR50 that solely function as scaffolding proteins.

**Formation and regulation of the complex**

Formation of the GPR50/TßRI complex adds a further dimension of the regulation of TßRI signaling, which is likely to happen in a cell context-dependent manner. Whereas expression of TßRI is widespread, the expression pattern of GPR50 is more restricted. Expression of GPR50 has been mainly studies in the brain and identified in the pituitary, the dorsomedial hypothalamus, tanycytes, the median eminence and the CA4 region of the dentate nucleus of the hippocampus (Batailler et al, 2011; Gubitz & Reppert, 1999b; Hamouda et al, 2007a; Sidibe et al, 2010). Expression of GPR50 in peripheral tissue is less well documented. The GPR50 mRNA has been observed in eye, testis, kidney, adrenal, intestine, lung, heart, ovary and skin (Drew et al, 2001). In addition, GPR50 expression has been shown to be highly regulated during different developmental stages with highest expression at E18 (Grunewald et al, 2012).

With regards to known expression of GPR50 and putative *in vivo* occurrence of the crosstalk, TGFβ signaling and TβRI expression has been observed in the *median eminence* (Prevot et al, 2010; Prevot et al, 2000), a region with high GPR50 levels, and might have an impact on the regulation of hormones implicated in reproduction. TGFβ signaling has also been reported to

specifies axons during brain development (Yi et al, 2010). As GPR50 is highly expressed in late embryonal stages (Grunewald et al, 2012) and is implicated in neurite outgrowth (Grunewald et al, 2009), this crosstalk might be of relevance during brain development or in synaptic plasticity of the adult brain.

Expression of GPR50 seems to be highly regulated. Significant variation of GPR50 expression has been observed depending on the photoperiod (Barrett et al, 2006), the energy content of the diet and the nutritional status (fed/fasted) of the animal (Ivanova et al, 2008). Proteolytic cleavage of the C-tail of GPR50, as reported recently, might be another way to regulate constitutive activation of TßRI (Grünewald et al, 2009; Li et al, 2011) as the truncated GPR50ΔCter construct was devoid of any effect on TßRI function.

Differential action of regulators such as GPR50 in time and space is likely to add to our understanding of how the cellular context determines the response to TGFβ.

Little is known about modified expression levels in cancer tissues. With regards to cancers, some studies detected an upregulation of GPR50 in early cancer states or tumorigenic tissue in pancreatic neoplasia (Buchholz et al, 2005) and nicotine-induced cellular transformation (Bavarva et al, 2013). Our results show that GPR50 might have a beneficial effect on tumor size and growth as shown in the xenograft experiments. Specific assays should help in the future to decipher the precise effect of GPR50 on tumor growth. Furthermore, the study of breast cancer microarrays/expression data will help to gain information about expression levels of GPR50 in tumor tissue.

**Revelation of first functional differences of the two frequent GPR50 variants**

Genetic association studies identified two common GPR50 variants, GPR50wt and GPR50Δ4, in the general population (Thomson et al, 2005). The GPR50 gene is located on the X

chromosome, in the Xq28 region. The GPR50Δ4 variant is associated with a higher risk for bipolar affective disorder (BPAD), major depression disorder (MDD) especially in females (Thomson et al, 2005). In another study, the GPR50Δ4 variant was associated with higher blood levels of fasting triglycerides and lower HDL levels (Bhattacharyya et al, 2006). Up to date no functional differences have been reported between the GPR50wt and GPR50Δ4 variants. Our study provide a first hint for the existence of such functional differences, which seem to be directly related to structural elements such as the $^{495}$ATSHP$^{499}$ motif located in close proximity to the $^{502}$TTGH$^{505}$ insertion/deletion. The ATSHP motif corresponds actually to one out of seven similar repetitive motifs within the C-terminal domain of GPR50 (Dufourny et al, 2008). Similar motifs are found in the C-terminal repeat domain of the RNA polymerase II, that functions as scaffold for auxiliary transcription factors in a phosphorylation-dependent manner. A similar function can be postulated for the GPR50 repeats, which is possibly modulated depending on the presence of the $^{502}$TTGH$^{505}$ insertion/deletion motif.

**Crosstalk between GPCRs and TßR - modulatory function of orphan GPCRs**

Previous studies showed that stimulation of proteoglycan synthesis in vascular smooth muscle cells by the thrombin receptor (PAR-1) requires TßRI kinase activity and Smad2 phosphorylation suggesting a possible transactivation mechanism between these two receptors. The authors did exclude an effect of PAR-1 on TGFß release but apart from that were unable to define the precise level of crosstalk at the origin of this effect. The GPR50/TßRI complex is the first example of a previously unrecognized crosstalk between the TGFß receptors and GPCRs at the receptor level. The capacity of GPCRs to engage into molecular complexes with other receptors, either of the same family (GPCR heteromers) or with proteins of other receptor families or transporters is increasingly recognized. Indeed,

such complexes significantly diversify the repertoire of pharmacological targets with a limited number of proteins. Such complexes might be of particular importance for orphan GPCRs. There exists indeed more than 100 orphan GPCRs for which no ligand has been identified yet. Apart from the ligand-dependent function that still have to be elucidated, an alternative hypotheses based on the existence of ligand-independent functions of orphan GPCRs is emerging (Levoye et al, 2006c). This also applies to GPR50, which has been shown to heteromerize with the melatonin $MT_1$ receptor and to inhibit ligand binding, G protein coupling and ß-arrestin recruitment to $MT_1$ in the common GPR50/$MT_1$ complex (Levoye et al, 2006a). As in the GPR50/TßRI complex, the C-terminal domain of GPR50 appears to play an important role in the modulation of the function of the interacting partner. Complex formation with TßRI clearly expands the idea that GPR50 is a co-receptor fine-tuning the function of other receptors with known function. These functions might be of particular importance for the evolutionary conservation of GPR50, which is likely to be a true orphan without ligand, as GPR50 orthologs in non-mammalian species bind melatonin, a property that has been lost in the mammalian GPR50 (Dufourny et al, 2008).

**6) Potential relevance for other members of the TßRI family**

In our study we used the prototypic type I receptor TβRI, which is one of seven type I receptor family members. Though the TβRI was the only member of the TßRI family identified in the TAP assay, we cannot exclude that GPR50 interacts with other type I receptors as our results might be biased by the expression levels of other TßRI members in HEK293T cells.

The other six members, four activin-like-receptors and two BMP-receptors, display strong sequence homology, underlie the same activation modus and were also found to bind FKBP12 (Wang et al, 1994). Alignment of the expression patterns of GPR50 and the other members of

the TßRI family, which are more restricted as TßRI might help to identify tissues of interest in the organism where a crosstalk might be of physiological relevance.

In conclusion, we describe here a new molecular complex composed of an orphan GPCR and TßRI that renders TßRI constitutively active towards the SMAD2/3 and the p38 pathways by dissociating the negative regulator FKBP12 from TßRI and most likely stabilizing an activated state of TßRI in the absence of TßRII. Such a constitutively active complex might be of interest in the context of the cell migration promoting and tumor inhibiting effect of TGFß.

## EXPERIMENTAL PROCEDURES

### Cell culture

HEK 293T, HeLa and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle medium (GIBCO) containing 8% fetal bovine serum (GIBCO) and 2% penicillin/streptomycin (GIBCO). Selective Medium for maintenance of MDA-MB-231 clones contained 250 µg/ml Geniticin (G418) (Sigma Aldrich). SNU638 cells were cultured in RPMI-1640 medium (GIBCO) with 10% FCS and 2% pen/strep.

### Reagents and antibodies

*Reagents:* FK506 and SB431542 were purchased from Sigma Aldrich and recombinant TGFβ-1 was used from Peprotech.

*Antibodies:* Phospho-SMAD2, phospho-SMAD3, p38 and phospho-p38 came from Cell Signaling, SMAD2/3 was used from BD Biosciences, anti-myc (A14 and 9E10), -TβRI V22, -Lamin (M20), and -GPR50 came from Santa Cruz Biotechnology. Mono- and polyclonal Flag-Antibodies were used from Sigma, HA- and GFP-antibodies were used from Roche. Anti-Tubulin was purchased from AbD Serotec. GPR50 antibody7 was produced by Kernov Antibody Services (Hamouda et al, 2007a). All antibodies were employed according to recommended dilutions for either immunoprecipitation or western blotting.

### Cell transfection and generation of stably overexpressing cells

Transient transfection was performed by using Lipofectamine® LTX reagent (Life Technologies) in HEK 293T cells, Xtremegene® 9 (Roche) for reporter gene assay in HeLa cells and JetPRIME (Polyplus) reagent for MDA-MB-231 and SNU638 cells, each employed according to the manufacturer’s instruction. Cells were incubated for 48 hrs before experimental use. Stably GPR50 overexpressing cells were generated by JETprime

transfection of G418 resistant GPR50 plasmid, selective pressure was established by using conditioned DMEM medium with 1 mg/ml G418 (Sigma Aldrich)

**Plasmid mutagenesis**

Primers for point mutations were designed with the help of the Agilent QuikChange Primer Design program. Mutagenesis was performed by PCR with the Phusion High Fidelity Polymerase (Finnzymes, Thermo Scientific).

**Tandem affinity purification**

All purification steps were conducted at 4 °C in the presence of a protease inhibitor mixture (Roche Applied Science), 1 mm orthovanadate, and 2 mM NaF. Crude membranes were prepared from ~$2 \times 10^8$ HEK 293 cells and solubilized overnight in solubilization buffer (75 mM Tris, 2 mM EDTA, 5 mM $MgCl_2$, pH 8.0) with 0.25% Brij96V at a concentration of 2 mg of protein/ml. The supernatant was recovered after centrifugation at 40,000 × g for 30 min and incubated for 4 h with 400 µl of rabbit IgG-Agarose (Sigma). The resin was washed three times with 1 ml of solubilization buffer, resuspended in 500 µl of the same buffer, and incubated overnight with 100 units of TEV protease (Invitrogen). The supernatant was collected, mixed with 500 µl of calmodulin buffer (75 mM Tris, 5 mM MgCl2, 50 mM $CaCl_2$, and 0.25% Brij96V, pH 8.0) and incubated for 2 h with 100 µl of calmodulin beads (Stratagene, La Jolla, CA). Beads were washed three times with 1 ml of calmodulin buffer and two times with 1 mL calmodulin rinsing buffer without detergent (50 mM ammonium bicarbonate, 2 mM $CaCl_2$) and resuspended into 100 µL of 50 mM ammonium bicarbonate, pH8.0. Perform trypsin digestion directly on beads by adding 1 mg of trypsin overnight at 37°C.

**Cellular lysis, co-immunoprecipitation and western blotting**

For preparation of cellular lysates, cells were harvested after transfection and stimulation according to protocol, in TNMG - Buffer with 0,5% NP-40 and a cocktail of protease inhibitorsd (Prunier et al, 2001) for 15 min, centrifuged at maximum speed for 20 min and supernatants were kept. Samples containing 500 µg to 1 mg protein were subjected to immunoprecipitation by incubating for 3 hrs with 2 to 5 µg of antibody. Protein G beads (Sigma), to enrich precipitates, were added for additional 2 hrs, prior to three washing steps in a Tris-EDTA-Magnesium buffer with 0,05% NP-40 buffer. Samples were diltuted in 2x Laemmli with 4% SDS and heated for 5 min at 95°C preceding SDS-PAGE. Cell Lysates for protein analysis were obtained after lysis with TNMG buffer, protein estimation was performed with BCA Assay (Thermo Scientific), 20 to 100 µg of sample were prepared and 4x SDS-Laemmli was added. Samples were heated at 95°C and separated on a 12% SDS Gel. Proteins were blotted on a PVDF membrane (Dutscher), blocked and incubated over night with in 3% milk or BSA-solution antibody. Incubation with fluorescence coupled secondary antibodies enables readout on an Odyssee reader.

**BRET analysis**

HEK 293T cells were transiently transfected in 6 well plates with 100 ng or 100 to 2000 ng respectively of the corresponding Luciferase- and YFP-coupled plasmids, grown over night and transferred in to 96-well-Optiplates (PerkinElmer Life Sciences), pre-coated with 10 µg/ml poly-L-lysine (Sigma), where they were grown for additional 24 hrs, washed with PBS, coelenterazine (Molecular Probes) for Luciferase stimulation was added and cells were subjected to measurement of emission at Luc and YFP wavelength on a Berthold Mithras™ as previously described (Maurice et al, 2010).

**Nuclear Extracts**

HEK 293T cells were seeded in 100 mm culture plates and transfected with mock, SMAD3 alone or co-transfected with GPR50wt and GPR50ΔTTGH variant. The cells were starved for 16 hrs in DMEM media without FBS and stimulated with 2 ng/mL of TGF-β for 2 hrs. The culture plates were rinsed twice with ice-cold phosphate-buffered saline (PBS). 500 µL of hypotonic buffer containing 1% NP-40 was added to each culture plate, and allowed to swell on ice for 15 min. The cells were scraped and taken into fresh eppendorf tube. The lysate was vortexed for 10 seconds, and the nuclei were pelleted (14000 rpm for 1 min). Supernatant was collected which is a cytoplasmic fraction. The nuclear pellets were resuspended in 100 to 200 µL of hypertonic buffer and rotated for 30 min. at 4°C. This extract was then centrifuged (13000 rpm for 20 min), and supernatant was collected which is nuclear fraction. The amount of protein was estimated with a BCA estimation kit. The buffer compositions were as follows. (i) Hypotonic buffer contained 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 25mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 mM dithiothreitol (DTT) (ii) Hypertonic buffer contained 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM EDTA, 25mM β-glycerophosphate, 1 mM $Na_3VO_4$ and 1 mM DTT. To both, buffers protease and phosphatase inhibitors were added just before use.

**Reporter gene assay**

HeLa cells were seeded in 12-well-plates transfected with the SMAD2-dependent Activin-response-element (ARE) Firefly Luciferase together with the FAST-myc co-factor, or the SMAD3-dependent CAGA Firefly luciferase reporter gene, increasing amounts of GPR50 ΔTTGH or WT and Renilla Luciferase as internal standard. Cells were left for 24 hrs, starved and stimulated with 2 ng/ml TGFβ overnight. Lysis and measurement were performed with

the Dual Luciferase Kit (Promega) according to manufacturer's advices. Experiments were performed in triplicates, figures show representative experiment.

**Radioligand binding assay**

MDA-MB-231 cells were plated in 6-well plates in incubated with $^{125}$I-TGFß (100000 cpm/mL; NEX267) in DMEM, 20 mM Hepes, pH 7.4, 0.4% BSA for 4h at 4°C to determine the number of surface exposed receptors. Non-specific binding was determined in the presence of a 100 fold excess of unlabled TGFß. Cells were washed twice with ice-cold PBS and extracted in 1mL 1N NaOH and $^{125}$I-TGFß quantified in a scintillation counter.

**Soft agar assay/anchorage-independence assay**

35mm dishes were coated with a layer of 0,5% Agar containing a DMEM/7% FBS solution. $1*10^5$ MDA-MB-231 cells were mixed with 0,3% agar containing DMEM-Media with 7% FCS and distributed upon the first layer. Colony formation was measured about 20 days after seeding.

**Migration and wound healing**

25.000 cells were seeded into 35mm µ-dishes with a silicon insert (IBIDI), starved over night and the insert was removed the following day. Closure was assessed every 6 hrs for 36 hrs under a light microscope. Analysis of wound closure was performed using ImageJ.

**Xenograft experiments**

$1x10^6$ MDA-MB-231 cells were diluted 1:1 in a Matrigel™ (BD Biosciences) and subcutaneously injected into the right and left flank of nude mice. Tumor growth was

monitored every 3 days and measured using a caliper. Tumor volume was calculated with the $(width)^2$ x length x $\pi/6$ formula.

**Statistical Analysis**

Statistical analysis of data was performed with a two-tailed unpaired T-Test. A p value $<0.05$ was considered for statistical significance.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures that can be found with this article.

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Authors contributions:

S.W. participated in the project design, performed most of the experiments, interpreted the data and contributed to the writing of the manuscript; Z.B.-C. designed and participated in the soft agar and xenograft experiments and interpreted the data; R.A. designed and performed nuclear translocation assays ; A-S.J. designed and performed all BRET experiments; A.D. designed and performed TAP experiments ; J-L.G. contributed to the management and to the work with MDA-MB-231 cells; A.S. performed experiments in the inital phase of the project;

N.C. conributed to the management in the initial phase of the project; T.C. performed control experiments; K.T. generated TßRI fusion proteins for BRET experiments; P.D. contributed to the management and design of the project. C.P. contributed to the management and design of the project, manuscript writing and performed control experiments in MDA-MB-231 cells; R.J. was responsible for the project supervision, experiment design, data interpretation, and manuscript writing and provided funding support.

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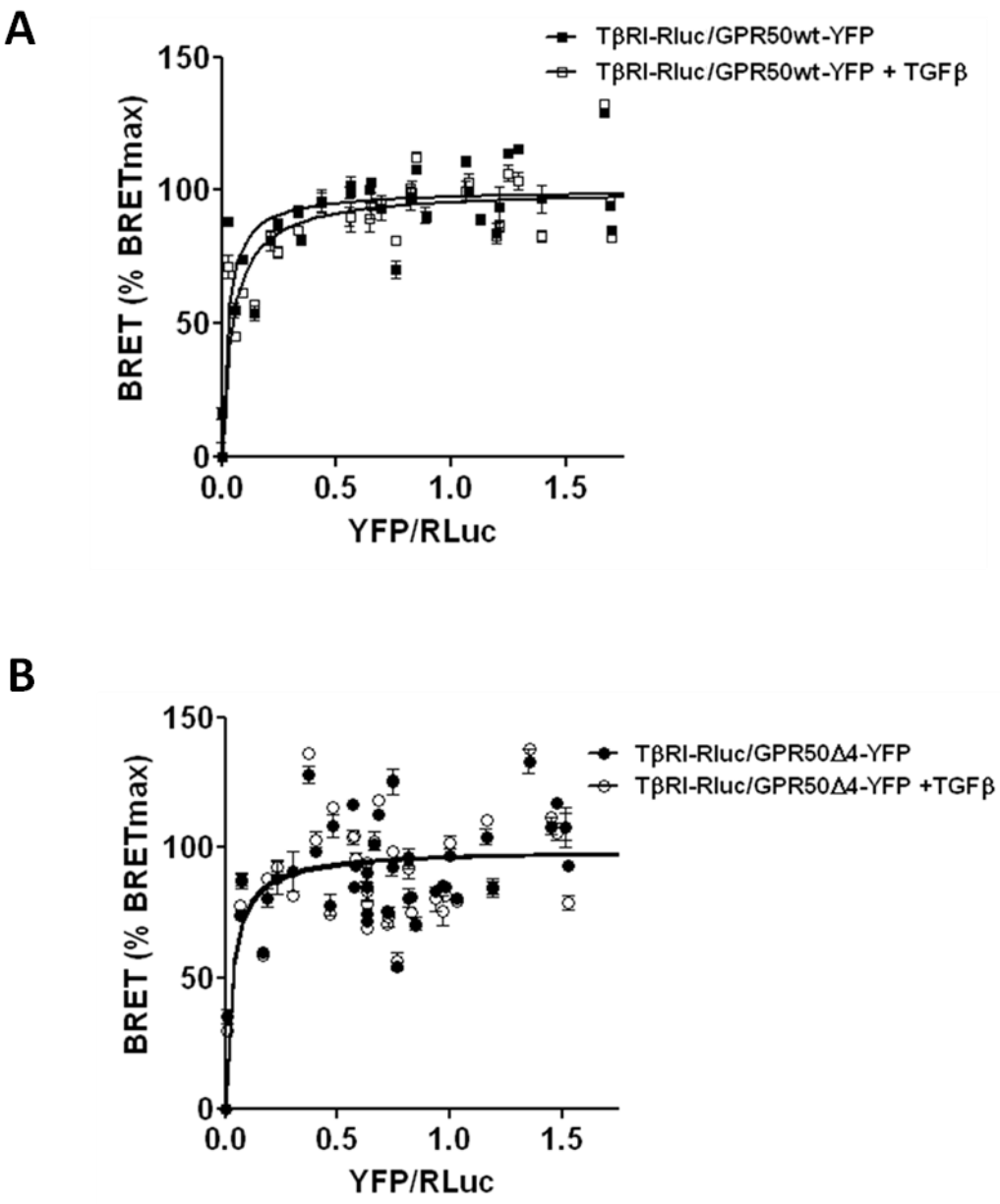
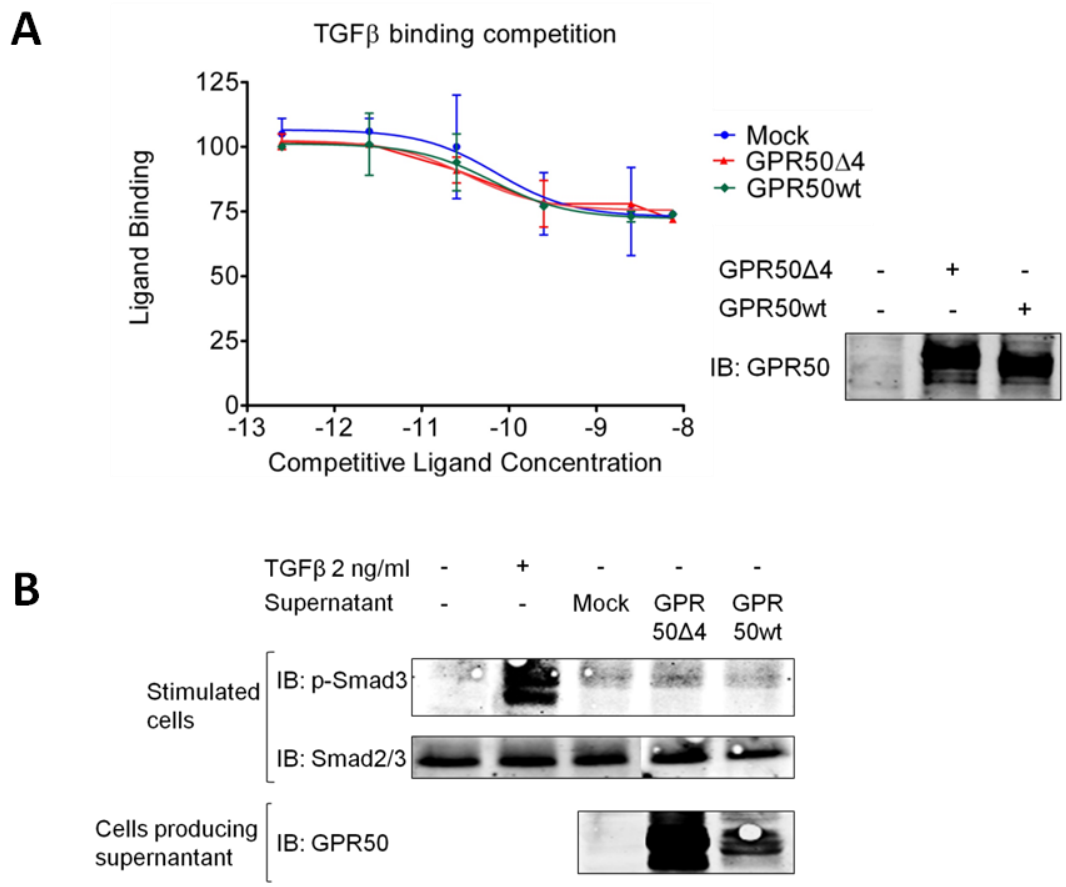
**Supplementary Figures**

Supplementary Figure 1




**S1 BRET experiments in absence and presence of ligand**
(A and B) For BRET saturation curves, HEK293T cells were transfected with constant amount of TβRI-Luc GPR50Δ4-YFP (A) or GPR50wt-YFP (B). Cells were washed and coelenterazine was added. BRET signals were measured after addition of 2 ng/ml of TGFβ or PBS as control.

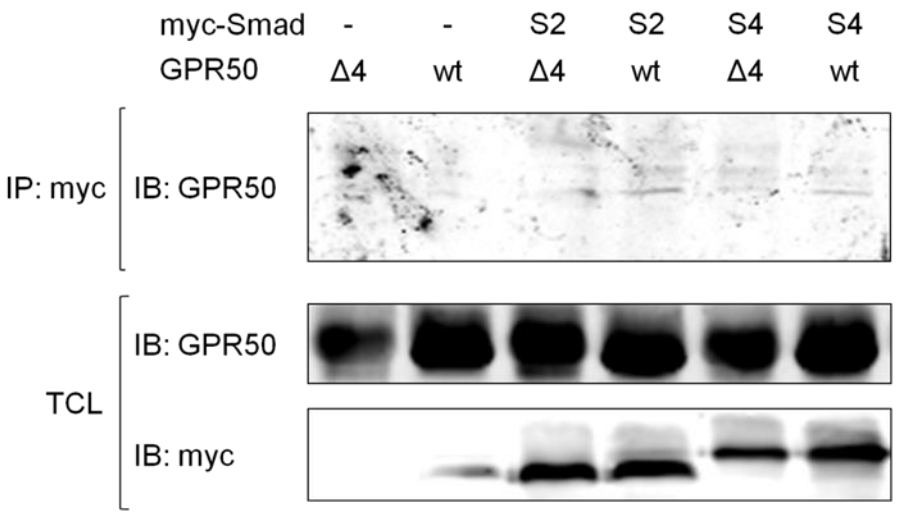
## Supplementary Figure 2




**(A) The effect of GPR50 on ligand binding**
To address binding of TGFβ, MDA-MB-231 cells were transfected with GPR50Δ4 and GPR50wt. Binding of TGFβ was assessed in a competion-radioligand-binding assay with radio-labeled and cold TGFβ.

**(B) Secretion of TGFβ in presence of GPR50.**
HEK 293T cells were starved and treated for one hour with the supernatant of HEK293T cells either transfected with Mock, GPR50Δ4 and GPR50ΔWT. Stimulation with 2ng/ml of TGFβ served as positive control. Cells were than analysed for their SMAD3 phosphorylation and total SMAD3. Cells providing supernatant were checked for GPR50 expression.

## Supplementary Figure 3




**S3 Co-IP of Smad2 and 4 with GPR50**
Co-immunoprecipation was performed with HEK293T cells co-transfected with myc-SMAD2 or SMAD4 and either GPR50Δ4 or GPR50wt. Lysates were incubated with an anti-myc antibody to precipate the SMADs. Precipitates were blotted against GPR50 and total lysates were analysed with an anti-myc and an anti-GPR50 antibody for plasmid expression.

# III. DISCUSSION

The initial aim of this project was to identify further binding partners for GPR50 by applying the TAP assay to the full-length receptor. After having identified the TβRI as a candidate binding partner, we successfully validated this interaction *in vitro* with different techniques. This prompted us to focus our attention on TβRI-dependent signal transduction and to investigate the impact of GPR50 on TGFβ signaling. Unexpectedly, we observed that GPR50 has the capacity to induce a ligand-independent activation of the TGFβ pathway, which is dependent on the TβRI kinase activity. The analysis of the molecular mechanism revealed that GPR50 competes with the negative regulator FKBP12 that keeps TβRI in an inactive conformation upon TβRI binding. Furthermore, we showed an activation of the TβRI in the absence of TβRII. The utilization of the MDA-MB-231 breast cancer cell lines overexpressing GPR50 or not, revealed pro-migratory and anti-proliferative effects of GPR50 that resembled the action of TGFβ in this model.

We describe here a new regulatory mechanism of TβRI-dependent signaling upon complex formation with the orphan 7TM protein GPR50 with a possible role in breast cancer development. These findings can be integrated into the current knowledge under different aspects and give rise for future research, which are depicted in the following:

## 1. The complex of TβRI and GPR50

This work identified a novel regulator of TGFβ signal transduction. The TGFβ signal transduction pathway is a direct pathway that goes straight from the membrane through the cytosol into the nucleus. The core components of this pathway are the TGFβ ligand, the serine/threonine kinase transmembrane receptors type I and type II and the signaling mediators, the Smads, that shuttle between cytosol and the nucleus, where they regulate gene expression (Massague, 1998). In order to establish signaling specificity and to allow cell context-specific signal transduction, proteins that interfere in a positive or a negative manner with activation or longevity of TGFβ signaling are necessary (Schmierer & Hill, 2007). During recent years, many proteins that modulate TGFβ signaling on the level of ligand (Moustakas & Heldin, 2009), receptor (Runyan et al, 2006) or the Smads (Xu et al, 2012) have been identified. With the discovery of GPR50 as a novel regulator of TGFβ signaling, we not only identified another protein, that can fine-tune TGFβ signaling, but that also adds previously unappreciated aspects to TGFβ-dependent signaling.

### 1.1. A new mode of activation for the TβRI

The dogma for activation of the TGFβ-dependent signaling consists in a well-defined signaling flow. The TGFβ ligand binds to the TβRII, that recruits the TβRI into the ternary complex (Wrana et al, 1994) and activates TβRI via phosphorylation in its GS domain (Wieser et al, 1995). Subsequently, TβRI phosphorylates the Smads that transduce the signal into the nucleus and regulate gene expression (Nakao et al, 1997b) or may alternatively activate other non-canonical pathways as p38 (Zhang, 2009). Studies have proven the necessity of the presence of the TβRII for the activation of TβRI (Luo & Lodish, 1996; Okadome et al, 1994; Vivien et al, 1995) due to the transphosphorylation of the GS domain of TβRI by TβRII (Carcamo et al, 1995). Our findings in contrast, demonstrate a previously undescribed mode of activation for the TβRI, independent from ligand binding and TβRII-dependent phosphorylation. The question is, how GPR50 is able to induce the kinase activity of the TβRI without the normally preceding steps.

#### 1.1.1. Competition of GPR50 for receptor binding with FKBP12

Our work could identify, that GPR50 competes with FKBP12 for TβRI binding. FKBP12 has been identified as negative regulator of TGFβ signaling with a gate-keeping function. It stabilizes an inhibitory confirmation of the TβRI by promoting the formation of an inhibitory wedge of the GS region and thus prevents ligand-independent signaling (Wang & Donahoe, 2004) by locking the TβRI in a kinase-inactive conformation and blocking the access for Smad binding to the GS region. Ligand binding and subsequent phosphorylation by the TβRII is accompanied by dissociation of FKBP12, although it remains unclear, at which point the release of FKBP12 occurs. Removal of FKBP12 leads to a loss of the inhibitory wedge in the GS domain and favors TβRII-mediated phosphorylation of Ser/Thr residues in the GS region, which allows the binding of the Smads and further signal propagation. Thus, the depletion of FKBP12 from the receptor might explain how GPR50 is able to induce a ligand-independent signaling. But there are two points that indicate that the release of FKBP12 is not the only event that happens in the presence of GPR50:

First of all, by release of FKBP12, we only observe a weak basal signaling activity that increases further upon ligand stimulation (Charng et al, 1996). This stands in contrast with the high levels of activation we observed in the presence of GPR50, that did only show a small further increase of Smad phosphorylation after ligand stimulation (Figure 2 A,B), giving a first hint, that GPR50 might have further effects on the TβRI. Different studies have shown,

that the reason for leaky signaling in the absence of FKBP12 is a hypersensitivity to TβRII (Chen et al, 1997; Stockwell & Schreiber, 1998). In cells deficient in TβRII, an activity of TβRI mutants incapable for binding the FKBP12 was only observed after ectopic expression of a wildtype TβRII but not kinase inactive TβRII mutants. Hence, leaky signaling in the absence of FKBP12 is explained by incidental ligand-independent phosphorylation in the GS domain by the TβRII. In contrast, our results show ligand-independent signaling in the presence of GPR50 in TβRII-lacking SNU638 gastric cancer cells. Thus we can suggest an active participation of GPR50 through the stabilization of a constitutively active conformation of the TβRI that does not require the TβRII anymore. With regards to literature, there exist some hints, that a constitutive activity of the TβRI is possible.

### 1.2. Constitutive activity of the TβRI and ligand-independent signaling

Currently, there is only little evidence that the human wildtype TβRI can be activated and phosphorylated by TβRII in the absence of ligand. Nevertheless, some cases of constitutive activity of the TβRI have been reported.

#### 1.2.1. The TβRI T204D mutant

In a study, that claimed to investigate the different effects of mutations in the GS domain on activity and activation of the TβRI (Wieser et al, 1995), a mutation of Thr204 into Asp, resulted in a ligand-independent constitutive activitation of the receptor. Thr204 is located adjacent to the GS domain. Although it is most likely not directly phosphorylated it promotes phosphorylation of Ser/Thr residues in the GS domain. Exchange of Thr into a more spacious and negatively charged Asp might results in conformational changes that translate to the GS domain. The GS domain probably loses its inhibitory wedge and is able to exert positive allosteric effects on the kinase domain. However, this mutant is still sensitive to the TβRII and signaling increases further in the presence of ligand and intact TβRII. In the case of GPR50 and the TβRI, we did so far not determine, whether our results show a complete independence of the TβRII and if additional effects are due to ordinarily activated TβRI or whether GPR50 and TβRII can exert mutually additive effects on TβRI activation. Results obtained in SNU638 cells argue that the GPR50/TβRI complex is completely ligand-insensitive and that residual TGFβ effects are generated from classical TβRI/TβRII complexes in cells expressing both isoforms.

### 1.2.2. ACVR1 R206H and L196P mutants in *fibrodysplasia ossificans progressiva*

In the genetic disease *fibrodysplasia ossificans progressiva* (FOP) with a phenotype of exceeding and inappropriate bone growth, two different mutants are associated with constitutive ligand-independent type I receptor activation. These mutants are found in the activin A receptor 1 (ACVR1), another one of the seven family members of type I receptors, that binds the BMP ligand and signals via Smad 1, 5 and 8. In the case of the R206H mutant, the concerned amino acid is located, similar to the TβRI T204D mutant in proximity to the GS domain (Groppe et al, 2007), a conserved region among all the type I receptors. This mutant is displaying ligand-independent activation (Song et al, 2010) and shows reduced binding to FKBP12 (Groppe et al, 2011), which is similar to our results. This constitutive signaling in the absence of ligand results in permanent activation of BMP target genes even in the absence of external signals, explaining the excessive and undesired bone growth in these patients. Another mutant, which has been identified involves a Leu to Pro substitution at position 196, an amino acid directly located in the GS domain and important for the binding of FKBP12 (Ohte et al, 2011). This mutant also shows constitutive activity, probably due to the loss of FKBP12 binding.

It is noteworthy that, at least for the R206H mutant, constitutive signaling still depends on the presence of the TβRII (Le & Wharton, 2012) even though its kinase activity is not required for ligand-independent activity of the TβRI (Bagarova et al, 2013). Thus, a TβRII-mediated phosphorylation in the GS domain of the TβRI is not essential for type I receptor activation in the case of the ACVR1 R206H mutant. Nevertheless, the presence of TβRII might have a distinct role, like binding of other proteins necessary for activation. In the case of the TβRI/GPR50 complex, TβRII-dependent functions can probably be complemented by the presence of GPR50.

### 1.2.3. TβRII-independent signaling in *C.elegans*

Further evidence for the capacity of the TβRI to signal in the absence of an intact TβRII comes from evolutionary lower organisms like *C.elegans*. In this organism, the type I receptor corresponds to the DAF-1 protein (Georgi et al, 1990) that can signal together with DAF-4, the type II receptor homolog to transduce the signals of the DAF-7 growth factor. It has been reported, that this receptor is able to signal in the absence of DAF-4 kinase activity (Gunther et al, 2000), yet the presence of fully active DAF-4 further increases its signaling activity. Sequence alignment and comparison reveals, that DAF-1, compared to other type I receptors,

presents striking differences in the important region around the GS domain. Most of the phosphorylation targets are absent, the GS domain is reduced and also a Leu- and Pro-residue for a hypothetical FKBP12 binding are missing. These findings suggest that this altered sequence leads to a divergent structure of the GS domain that might result in a loss of the inhibitory wedge and its negative effects on the kinase domain of DAF-1, which eventually promotes constitutive activity. In contrast, the ligand-binding or at least the ligand-binding domain is required for this constitutive activity, suggesting either steric effects of the ligand binding domain or genuine ligand binding involved in the constitutive activity. This suggests, that at some point during evolution a constitutive active and ligand-independent type I receptor must have been useful. Probably, FKBP12 and the TβRII developed to provide control mechanisms for the TβRI activity that became necessary during evolution. Unfortunately it has not been studied whether, analogous to the ACVR1 R206H mutant, the presence of DAF-4 is required or whether DAF-1 can even signal in the absence of the type 2 receptor.

### 1.2.4. Stretch-induced activation of the TβRI

Recently, work has been published, that describes ligand-independent activation and signaling of the TβRI in case of cellular stretching (Chen et al, 2013). The mechanisms that act upstream and are responsible for activation have so far not been dissected, thus not excluding the involvement of other ligands than TGFβ. Furthermore, no information is given about the involvement of the TβRII in activation.

The existence of constitutive active forms shows that under certain circumstances, a constitutive activity and a ligand-independent mechanism must have been advantageous that probably got lost during evolution. Taken together, the existence of this constitutive active forms, either early in evolution, artificial in laboratory mutations or in disease genotypes, support in coherence with our findings, that a type I receptor can signal in the absence of ligand and the absence of type II receptor kinase activity. Additionally, the examination of the behavior of this mutants showed, that a TβRII-mediated phosphorylation after ligand stimulation can have further increasing effects, but that it is not compulsory for an activation of the type I receptor. For the moment we have not figured out, whether, in the presence of GPR50, a GS domain phosphorylation is necessary for signaling or whether the TβRI can signal just by the allosteric alterations provided by GPR50. Further studies in a receptor-

deficient environment will hopefully help to gain information about the circumstances of a GPR50-mediated constitutive activity of the receptor.

### 1.3. The active role of GPR50 in inducing constitutive TβRI activity

Our work consists of the novelty, that GPR50 is able to activate the TβRI in absence of the TβRII. An interesting task for the future will be the investigation of additional effects of GPR50 making TβRII unnecessary. This research can be based on several hypotheses:

#### 1.3.1. GPR50 acting as a kinase

GPR50 could be a kinase itself that phosphorylates the GS domain in order to induce kinase activity of the TβRI. Even though GPR50 has a characteristic long cytosolic C-terminus, modern *in silico* techniques of sequence alignment and motif identification did not identify any homology to any known kinase. Additionally, the C-terminus is an ancestor of the RNA Polymerase II, a nuclear protein without any intrinsic kinase activity.

#### 1.3.2. GPR50 acting as a scaffold protein

GPR50 might be a scaffold for another kinases that can phosphorylate TβRI. Our results did so far not include the demonstration of direct association of the TβRI and GPR50. Thus, the activation might also pass through an intermediate. It can be imagined, that GPR50 recruits another cytosolic serine/threonine kinase that is capable of phosphorylating the TβRI to explain its activation in the absence of the TβRII. But yet, neither our TAP-assay, nor a Y2H assay from our group or others identified any kinase as a potential binding partner for GPR50. And with regards to TβRI, there is no indication that the TβRI gets phosphorylated by any other kinase then a type II receptor in the GS domain. Further experiments will address, whether GPR50 is in direct contact with the TβRI or whether additional proteins are involved.

#### 1.3.3. Stabilisation of an active conformation of the TβRI

Our results indicate that GPR50 removes FKBP12 and stabilizes an active conformation of the TβRI. This observation is supported by the fact, that there is only a partial but sufficient competition between FKBP12 and GPR50. FKBP12 contacts the TβRI on two positions, with the 40s loop to the GS domain and with the 80s, which contains the ATGHP motif, also the L45 loop responsible for Smad binding (see Introduction, Chapter 3.2.3.4). We identified only the $^{84}$ATGHP$^{88}$ of FKBP12 80's loop for being similar in GPR50, what proposes a partial

competition with FKBP12, that is yet sufficient to provoke its release and is consistent with our data that a loss of the the HP residues in FKBP12 is enough to disturb the binding to the TβRI. GPR50 probably takes place at the interface of Smad binding (L45 loop) but additional structural features of GPR50 might provoke a loss of the inhibitory wedge in the TβRI GS domain that usually gets stabilized by the 40's loop of FKBP12. Probably, an available crystal structure or structural prediction of GPR50 might allow a comparison of FKBP12 and GPR50 and their binding sites to TβRI.

### 1.3.4. Phosphorylation status in the presence of GPR50

We did so far not examine the phosphorylation status of the TβRI in presence of GPR50. A mutation of all the known putative phosphorylation residues in the GS region at once prohibits TGFβ signaling, suggesting a role for downstream signaling (Wieser et al, 1995). An autophosphorylation in absence of the TβRII seems quite unlikely, even though it has been reported, that homodimers of the TβRI can complement each other if in one GS- and in the other the kinase domain are defective (Weis-Garcia & Massague, 1996). If we imagine GPR50 as an orchestrating molecule, that regroups TβRI and releases the inhibitory wedge in the TβRI, we cannot exclude an auto-transphosphorylation of TβRI within such clusters in the presence of GPR50. It will be an important question for the future, to identify the phosphorylation status of the TβRI in presence of GPR50 and to identify whether GPR50 still exerts its promoting effect on TβRI in the absence of known phosphorylation sites in the TβRI.

### 1.3.5. Importance of further FKBP12 similarity motifs

GPR50 contains four repetitive motifs, that are similar to the $^{84}$ATGHP$^{88}$ sites in FKBP12 (as shown in Figure 3e). For the moment, we do not know, whether they all have the same influence or whether the effects we observed are restricted to the $^{495}$ATSHP$^{499}$ sequence in proximity to the insertion/deletion of GPR50. The deletion might cause steric alterations that favor the interaction between TβRI and GPR50. Currently, it can not be excluded that all motifs regroup several receptors at once. Further mutational studies will surely provide insight into the role of this repetitive sequence in GPR50 with regards to TβRI activation.

### 1.3.6. Complex formation in the presence of GPR50

Another possibility is, to imagine GPR50 as a novel co-receptor for TGFβ that might create, in complex with TβRI, a high affinity binding site for TGFβ. It has been reported before that some receptors gain novel ligand binding capacities upon the formation of uncommon receptor combinations (ten Dijke & Hill, 2004). But according to our results, this hypothesis seems quite implausible. Our results show that the C-tail of GPR50 is involved in signal transduction and that the presence of the transmembrane part is not sufficient for downstream signaling. Thus, a role where GPR50 serves as a ligand-binding protein in the complex is difficult to imagine. Even though we cannot rule out the possibility that the different parts of GPR50 have separate functions: one that is responsible for the binding of a ligand and another that modulates the intracellular part to induce the activity of the TβRI, which is supported by the idea that full length GPR50 is required for being effective.

## 1.4. Regulation of complex formation GPR50/TβRI

In our case, the GPR50/TβRI complex seems to be formed constitutively. However, several ways to regulate this complex can be envisioned in a physiological context. According to existing principles in biology the following possibilities exist to regulate complex formation:

### 1.4.1. Regulation of cellular protein levels

*Protein expression*

The first possibility lies in the regulation of protein levels, either through gene expression, or half-life limitation by protein degradation. We know that TβRI is ubiquitously expressed, thus we can suppose its protein levels being more or less stable. With regards to GPR50, studies could show GPR50 expression levels are regulated during season (Barrett et al, 2006) and depending on the energy status (Ivanova et al, 2008). Furthermore, the existence of a miRNA target sequence in the promoter region (John et al, 2004) and the findings for a Dnmt-dependent regulation (Kotini et al, 2011) suggest a tight control of GPR50 expression and levels in the cell.

*Protein stability*

With regards to protein stability, other means of regulation of complex formation might consist in proteasomal or lysosomal degradation. Several mechanism that imply the ubiquitin-mediated degradation of the TβRI receptor complex have been described (Soond & Chantry,

2011) suggesting an involvement of such pathways to regulate the TβRI/GPR50 complex. GPR50, though being an orphan 7TM protein, might also be the subject to classical GPCR-desensitization pathways or regulations. The findings, that GPR50 is subject to proteolytic cleavage and subsequent nuclear translocation (Li et al, 2011) might add a possibility to regulate the activity of the complex, since the cytosolic part of GPR50 is indispensable for activation of the TβRI. Further investigation that addresses the proteolytic cleavage of GPR50 will surely provide more insight into possible regulation mechanisms of GPR50 activity.

*Protein localisation*

Regulation of the complex might also be established through a regulation of the subcellular localization of both proteins. It has been well described, that the TβRI is subject to internalization either in order to become recycled or in order to get lysosomally degradated (Di Guglielmo et al, 2003).

The interaction itself in contrast does not seem to interfere with localization of the TβRI; binding experiments in absence and presence of GPR50 did not show any changes in binding capacity, providing evidence that neither increased surface levels of receptor nor increased internalization or a change in receptor stability might occur for the complex in presence of GPR50.

### 1.4.2. Regulation by feedback loops

A quite frequent observation is, that most of the genes, that interfere with the activity of the TGFβ signaling pathway and its receptor are part of feedback-loop mechanisms. Negative regulators as Smad7 (Nakao et al, 1997a) and DRAK2 (Yang et al, 2012) are often induced by TGFβ signaling in order to attenuate signaling. This is a sophisticated mean of a cell to regulate signaling spatially and temporarily. For the moment, we did not investigate, if active TGFβ signaling has any influence on the expression of GPR50. But future experiments will address the question whether there is a connection between GPR50 expression and active TGFβ signaling.

Another question is, how the interaction itself deals with negative feedback loops. Usually, we would suggest, that the activation of the receptor in GPR50 goes along with an increased expression of negative feedback loop regulators as Smad7 that would terminate or weaken signaling by receptor complex degradation and a decrease in Smad phosphorylation. We did not address so far, whether GPR50 provides any protection against signaling arrest. Our

results do not indicate a decrease in signaling, as Smad phosphorylation remains constant. But we can assume, that there must be a way to cope with the habitual negative feedback loop that occurs after TGFβ signaling. It might be interesting to see whether there is a competition between Smad7 and GPR50 on receptor binding or whether GPR50 also possesses the capacity to prevent binding of the negative Smad7 regulator and subsequent arrest of signaling if necessary. In turn, a signal induced by GPR50 that can endure long time would provide the cell with a constant amount of active TGFβ signaling.

#### 1.4.3. Regulation by other proteins

Another possibility lies in the fact, that accessory proteins might induce the interaction by functioning as scaffold proteins. Thus, distinct signals might induce a protein-mediated juxtaposition of GPR50 and the TβRI with the purpose of allowing a GPR50-induced constitutive activation in the absence of ligand. Such regulation would be a rapid way to induce TGFβ-dependent signaling and is in favor of mechanism that involves gene expression of receptor and ligand and secretion.

### 1.5. Translation of our concept of activation to the TGFβ superfamily

In the present study, the main focus was lying on the prototypic TβRI/TβRII complex and its canonical downstream signaling through the Smads. After having concluded about the general aspects of complex formation and signaling mechanisms, an interesting task is now, to see whether this concept is applicable to the entire signaling spectrum of the TGFβ superfamily of cytokines.

#### 1.5.1. Extension to non-canonical signaling of receptors

The TβRI can not only signal via the Smads, but can also be directed to activate other downstream signaling pathways through non-canonical signaling (Zhang, 2009). Receptor associated proteins are often responsible for activating these pathways, as shown for TAK1 that activates p38 (Hanafusa et al, 1999) and TRAF6 that directs TGFβ signals towards p38 and JNK (Yamashita et al, 2008) (see also Introduction Chapter 3.3 and 3.4). Whether GPR50-mediated activation of the TβRI results in activation of multiple possible downstream signaling pathways or whether GPR50 selectively activates distinct signaling pathways downstream of the TβRI has not been thoroughly studied. Our results indicate that GPR50

promotes also activation of p38, suggesting a more general effect of GPR50 on TβRI signaling. But additional experiments will provide understanding for the influence of GPR50 on downstream signaling.

### 1.5.2. Extension to other TGFβ receptor family members

The TβRI is one of seven members of type I receptors of the TGFβ ligand superfamily. The other six members are four activin-like-receptors and two BMP-receptors. They display a strong sequence homology, especially in the cytoplasmic part that is relevant for signal transduction: they all comprise the GS domain and are capable for binding FKBP12 (Wang et al, 1994), suggesting that they underlie all the same activation mechanism, even though most information was gained upon investigating the TβRI and TβRII. Since our results suggest so far no mechanism, which seems to be unique for TβRI, the extension of this concept of activation towards other type I receptors seems likely. So far, with regards to the TAP assay, no further type I receptors interacting with GPR50, but this might depend on the relative expression levels of the different TβRI family members. One-by-one verification of these interactions by co-IP or BRET should resolve this issue and show whether activation of Smad 1,5 and 8 could be also regulated by GPR50. This in turn can also contribute to the understanding of the physiological relevance of such complexes (see below).

## 1.6. Crosstalk between GPCRs and TβRI

The proteins that regulate TGFβ signaling are quite divers, ranging from proteins with enzymatic functions (phosphatases, kinases, ubiquitin ligases) to multidomain proteins with adaptor functions (see Chapter I – 3.3.). So far, there have been no reports describing 7TM proteins interfering with TβRI activation through direct receptor interaction. Nevertheless, some intersections have been described concerning a crosstalk between GPCR- and TGFβ signaling at downstream levels during the last years.

It has been shown, that TGFβ signaling can be regulated by proteins that usually are associated with regulation of GPCR signaling. The β-arrestin molecule is, amongst others, responsible for GPCR internalization and signal termination. A similar mechanism was described for the accessory TβRIII betaglycan (Chen et al, 2003), where interaction induces TβRII/TβRIII receptor complex internalization and signaling arrest. This data got support by the detection of a direct interaction between the TβRII and β-arrestin (McLean et al, 2013). Another link has been found for GPCR-specific kinases, the GRKs, that phosphorylate a

GPCR prior to β-arrestin binding, thus being part of a negative regulating mechanism. Analogous events have been described, where GRK2 had been identified as TGFβ-response gene, which then associates with Smads and prevents their C-terminal phosphorylation, providing a mean for negative feedback loop regulation. Though these molecules have been identified originally to be responsible for the regulation of GPCRs it is now established, that their modes of action can also be extended to other signal transduction pathways (Gurevich et al, 2012) (Shenoy & Lefkowitz, 2011). Thus, in this case, we should not directly speak of a crosstalk, but of proteins with a regulatory function that initially have been identified in association with GPCRs, but are now employable for several other signal transduction pathways. Being an orphan receptor, no ligand-promoted pathway activation involving GRKs and β-arrestins have so far been described for GPR50.

Recent work from the group of Peter Little reported a crosstalk between the thrombin receptor (GPCR) and the TβRI, which represents the first example for such a crosstalk. The authors could show that ligand-dependent activation of the thrombin receptor PAR-1 can result in a TβRI-dependent activation of the Smads (Burch et al, 2010). This work provides first evidence of a crosstalk between GPCRs and the TβRI via transactivation on the receptor level (Burch et al, 2012). But so far a detailed mechanism, whether the crosstalk occurs directly through physical interaction on the receptor level or via other downstream intermediates that activate the TβRI, is missing. An indirect activation of a type I receptor has been shown once for serotonin-mediated activation of BMP-dependent Smads, that involve 5-$HT_{2B/D}$ and Rho kinases (Liu et al, 2009b). The present work of the GPR50/TβRI complex is supported by literature evidence, that TβRI-dependent signaling might be affected by GPCRs. In comparison to the thrombin receptor, it seems, according to the current state of knowledge, implausible that we also have a ligand-dependent transactivation. GPR50 seems to have direct effects on the receptor and act as direct regulator of the TβRI. Thus, we report here for the first time a physical association of the TβRI and a 7TM protein on the membrane level.

GPCRs have a tendency to form higher order complexes, either with other GPCRs or GPCR-regulating proteins (Maurice et al, 2011a; Maurice et al, 2011b), but also with other membrane receptors of the RTK family (Wetzker & Bohmer, 2003) or ion channels (Altier & Zamponi, 2011). A complex between the TβRI and GPR50 adds another element to the diversity of GPCR interactions and the crosstalk they can exert. Though we have to point out that a case of a constitutive, ligand-independent activation upon complex formation still stands out compared to the cases of ligand- or phosphorylation-dependent transactivation.

## 2. A new role for GPR50

With our findings of GPR50 forming a complex with the TβRI that induces its constitutive activation, we also established another role for the orphan 7TM protein GPR50. Initially, it was supposed, that transmembrane proteins can only have a function after activation by a ligand. The principal intention was their deorphanization, the discovery of their natural ligand. During the last decade, a slight shift from this doctrine arose with emerging findings proposing a ligand-independent function for orphan 7TM proteins (Levoye et al, 2006c). A prototypic example is GPR50, that has been associated with different ligand-independent functions that converge in the principle that GPR50 complex formation modulates activity of its binding partners, either in membrane, upon hetermerization with $MT_1$ (Levoye et al, 2006a), in the cytosol with Nogo-A (Grunewald et al, 2009) or upon partial proteolytic cleavage of the C-Tail in the nucleus with TIP60 (Li et al, 2011). Our findings provided another example for this ligand-independent function, that reinforce the concept of GPR50 being an interacting and activity-modulating protein. In contrast to existing publications, that identified Nogo-A and TIP60 as interacting partners, that were based on a Y2H assay with the C-terminal cytosolic portion of GPR50, we used a technique to identify proteins, that interact with the full-length receptor. The results of our TAP assay will probably help to identify further membrane proteins that might be regulated upon complex formation with GPR50.

### 2.1. Mechanism of function of GPR50

Further information about the of GPR50 and its regulation will surely also help to identify the conditions of these complexes. As GPR50 is most likely a product of genetic fusion of the *Mel1c* gene and an ancestor of the RNA polymerase II (Dufourny et al, 2008), it might also be divided in different functional units. The work from Li *et al.* concerning TIP60 provided evidence for a proteolytic cleavage with a subsequent nuclear translocation of the C-terminal part, which is consistent with the nuclear localization of the RNA Pol II. It will be an interesting task, to unravel the mechanism that induce the proteolytic cleavage of GPR50, thus proposing an important mode of regulation of its activity via its subcellular localization. On the one hand, we might suppose, that the transmembrane part and the cytosolic part function independently as a result of their different origins. But the results from our laboratory for $MT_1$ and the TβRI indicate a requirement for transmembrane and cytosolic part for being effective.

It is quite intriguing to see, how evolution was able to construct something functional by the fusion of two proteins, that probably originally had independent functions but obtain unique properties upon their combination. Further work will hopefully provide more clear information about the function of the different parts of GPR50, their association and proteolytic cleavage of GPR50, which might in turn also affect the complex formation and stability with the TβRI.

## 2.2. GPR50 activity

### 2.2.1. A ligand for GPR50?

An important remark must be made that over all we should be aware of the fact that (1) though it seems unlikely, we have to take in account that GPR50 might have an endogenous ligand that remains to be identified. Although currently, beside the proven inability for melatonin binding, no other possible ligand could have been identified. By employing *in silico* based methods and analysis of structural data of GPR50 a possible ligand might be identified. Under such conditions, we could also hypothesize a transactivation of the TGFβ signaling pathway by GPR50's ligand, as for example shown for serotonin receptors 5-$HT_{2B/D}$ and the BMP signaling pathway (Liu et al, 2009b). Especially in circumstances of overexpression, receptors often become hypersensitive to their ligands. But with regards to its restricted expression pattern, it seems not really likely, that a GPR50 ligand will be secreted or produced by HEK293T-, HeLa-, MDA-MB-231- and SNU638 cells that were used in our studies

### 2.2.2. Constitutive activity of GPR50?

Another possibility is the one of constitutive activity of GPR50. Being a 7TM protein, it might be possible, that GPR50 is constitutively coupled to G proteins, as it has been demonstrated for certain orphan GPCRs as GPR26 and GPR78 (Jones et al, 2007). Thus, an activation mechanism of GPR50 referring to "classical" G protein signaling cannot be completely excluded even so convincing evidence is currently lacking.

### 2.2.3. GPR50 as scaffold protein

As already pointed out above, the discovery of numerous putative interacting partners for GPR50 supports its function as a scaffold protein that recruits proteins which are responsible

for the effects we observe in the presence of GPR50. A function as a scaffolding protein, at least of the heptarepeats in the cytosolic part of GPR50, would be in accordance with the known function of the hepta-repeat motifs identified in the RNA pol II that can recruit a divers panel of other proteins.

### 2.3. Homology of GPR50 and FKBP12

During the dissection of the molecular mechanism of the interaction of GPR50 and the TβRI, we identified GPR50 amino acid motifs that are similar to a C-terminal motif in FKBP12. The question that arises is whether this motif in GPR50 might also conduct other functions that FKBP12 is associated with. The amino acids H87 and P88 of the 80s loop in FKBP12 are exposed, thus forming an interaction surface with other proteins as for the calcineurin phosphatase (Aldape et al, 1992; DeCenzo et al, 1996; Futer et al, 1995). In addition FKBP12 also regulates the activity of ryanodine- and IP3- calcium channels that trigger calcium release fom intracellular stores (Ivery, 2000). In turn, GPR50 has been identified to be regulated in dependency of the amount of plasma membrane calcium channels that depend on the activity of internal calcium stores (Zagranichnaya et al, 2005).

The actions of immunophilins as FKBP12 seem also to play an important role in the nervous system (Snyder et al, 1998), a fact of interest in relation to the GPR50 expression pattern in the brain.

### 2.4. A network of GPR50 interacting partners?

Beside the numerous binding partners of GPR50, which have been identified via Y2H- or TAP assay, we count four studies that describe the mode of action for GPR50 as an interacting protein. An interesting question is, whether they might all converge at some point, like going hand-in-hand or whether they all function independently in a different context and under different circumstances. Additionally these interacting partners are sometimes proteins that have a precise expression pattern, thus suggesting that GPR50 exerts this function like a chameleon in a cell- and tissue-dependent context.

### 2.5. Comparison of GPR50 variants

Two frequent human variants exist for GPR50, the GPR50wt and GPR50Δ4 variant, with the deletion of $^{502}$Thr Thr Gly His$^{505}$ and a coupled amino acid exchange at position 532, with a frequency of 40% in the human population (Thomson et al, 2005). Though these variant have

been associated with different phenotypes in genetic association studies (see Introduction, Chapter 2.3.3.) as the tendency to develop mental disorders (Thomson et al., 2005) or lead to altered lipid parameters (Bhattacharyya et al., 2006), no study could so far provide any functional differences of both variants. Our study reports for the first time functional differences of both variants on the molecular level. First hints came up with the findings that both have a different strength to activate TβRI-dependent signal transduction. A deeper look revealed that one of the FKBP12 similarity motifs lies in direct proximity to the 4 amino acid $^{502}$TTGH$^{505}$ insertion/deletion and might be the reason for a higher power of the GPR50Δ4 to induce the ligand-independent activation of the TβRI. Molecular evidence was also provided by the result, that the $^{495}$ATSHP$^{499}$ motif is responsible for the competition with FKBP12 upon TβRI binding. The amino acids of the insertion/deletion at position 502 to 505, which contains two Thr residues might be subject to regulatory covalent modifications through phosphorylation. Addition of phosphate groups can cause structural changes effecting the conformation of the$^{495}$ATSHP$^{499}$ motif. How far our findings can provide explanation for the associated phenotypes, has to be examined in a more physiologic context in the future.

### 2.6. A new mode of action for orphan GPCRs

Though in the recent years, some ligand-independent functions for orphan GPCRs have been identified, for the moment they have been nearly exclusively restricted to heteromer formation with related GPCRs. Literature data that shows the regulation of other proteins is still rare: only the long-time orphan GPR37 has been described to interact with ion channels and the dopamine active transporter DAT (Marazziti et al, 2007) upon physical interaction. The complex of GPR50 and TβRI describes a new field of action for an orphan GPCR in the regulation of core signaling pathways upon the interplay with the TGFβ receptor serine/threonine kinase. Thus, GPR50 is part of the signaling network of a cell, which might be the case also for other orphan 7TM proteins. It will be interesting to see, whether we find similar mechanisms also for other signal transduction pathways as RTKs. For the moment, we still count about 100 orphan proteins that are homologous with GPCRs that wait for an elucidation of their cellular task.

## 3. Physiological importance of the TβRI-GPR50 complex

Our study mainly focused on the dissection of the molecular mechanisms that determine the interaction and its outcome in cellular models with exogenous expression. The future task is now, to identify the physiological importance of this crosstalk.

### 3.1. GPR50 and cancer

In order to have a first indication about the impact of GPR50 on TGFβ signaling in a functional context, we decided to overexpress GPR50 in MDA-MB-231 breast cancer cells. As the TGFβ signaling pathway is an important player in cancer development and progression (Massague, 2008), the MDA-MB-231 model appeared to be particularly attractive, as it is widely used to study the pro-migratory and anti-proliferative effects of TGFβ signaling. Accordingly, GPR50 induced cellular migration and decreased cellular proliferation *in vitro* and *in vivo*. It will be interesting to further investigate whether the diminished tumor growth in GPR50 expressing cells might be also related to a higher occurrence of metastatic events. TGFβ is known to promote metastasis into lung (Padua et al, 2008) and bone (Yin et al, 1999) in MDA-MB-231 cells. Thus, we might also imagine that cells with high GPR50 expression have higher metastasis rates. For these studies, appropriate experiments *in vivo* that monitor metastasis will provide more insight in the future. Models of spontaneous cancer development can give insight into GPR50's role in tumor formation: our intention is, to study the effect of GPR50 in MMTV/Neu transgenic mice (Taneja et al, 2009), that spontaneously develop breast cancer. A crossing of these mice with GPR50 KO mice can provide further *in vivo* evidence for the impact of GPR50 on cancer development. A protective effect of TGFβ signaling has already been described (Siegel et al, 2003).

Additionally, it will be interesting to genotype cancer patients according to their GPR50 variant. This could give us an idea whether one genotype has a higher risk for cancer development to put our differential data for GPR50Δ4 and GPR50wt in a physiological context. Unfortunately, there are only few databases which align cancer risk and genetic variants. But the recent findings about the importance of genetic variants and their different functionality will hopefully drive interest about the correlation of germline gene variants and disease predisposition as recently demonstrated for the melatonin $MT_2$ receptor and diabetes risk (Bonnefond et al, 2012).

Some of the positive regulators of TGFβ signaling are important tumorsuppressors. Thus, their upregulation could provide additional protective mechanisms against cancer development. There are two studies, which revealed a GPR50 upregulation in pancreatic neoplasms (Buchholz et al, 2005) and nicotine-induced transformation of lung cells (Bavarva et al, 2013). Possibly, an upregulation of GPR50 that goes along with a constitutive TGFβ signaling activity might provide a protective mechanism for a cell against cancer in early stages. In contrast, in late cancer stages, TGFβ signaling is either deficient or can have tumorpromoting effects. In a progressed cancer, an upregulation might be of negative influence, thus constant TGFβ might increase the metastatic potential of a cell. A screening of human cancers concerning altered GPR50 expression can provide more insight for the role of GPR50 in cancer development.

SNU638 cells are cancer cells that have a mutation in the TβRII gene leading to the expression of a truncated extracellular variant (Ku & Park, 2005; Myeroff et al, 1995). Our results in SNU638 cells have shown that ectopic expression of GPR50 can restore TβRI-dependent signaling in these cells. These findings are promising and bear therapeutic potential for gene therapies of cancer with mutant TβRII or TGFβ ligands.

In contrast, for late tumor stages, constitutive activation of TβRI might be undesired and should rather be blocked by appropriate pharmaceutical means.

### 3.2. Relation to known functions and the expression pattern of GPR50

Compared to the TβRI, GPR50 seems to have a more restricted expression pattern. Currently, most available data concern GPR50 expression patterns in the brain, where it has been found in several regions, comprising the area around the 3$^{rd}$ ventricle of median eminence and the tanycytes, hypothalamic regions as the DMH and other regions with different functions. Only few studies investigated the expression of GPR50 in peripheral tissue, demonstrating that GPR50 mRNA is present in various tissues as heart, kidney, testis, liver (Drew et al, 2001). KO mouse models will hopefully provide more information about tissues that contain GPR50 proteins. In addition, compared to the TβRI, the expression of GPR50 seems to be subject of regulation as it has been demonstrated for a season- and energy-status amount of GPR50 in the brain (Barrett et al, 2006; Ivanova et al, 2008). In order to find regions where the crosstalk might take place *in vivo*, tissues that express both receptors will help to gain information.

### 3.2.1. GPR50 expression in the brain

A region with a markedly high expression of GPR50 are tanycytes, that surround the 3rd ventricle (Sidibe et al, 2010). The TβRI is also expressed in this brain area (Bouret et al, 2004; Bouret et al, 2002; Prevot et al, 2010; Prevot et al, 2000) and its expression can be induced by the gonadotropin-releasing hormone GnRH and subsequently downregulates GnRH activity. Probably, the GPR50/TβRI interaction might occur under certain conditions in order to orchestrate reproductive actions in organisms.

Furthermore, the expression of GPR50 displays different levels during embryogenesis, with the highest amount in late embryonal phase at E18 (Grunewald et al, 2012), when complex structures are formed and the development of brain compartments and neuronal connections takes place. This is coherent with the observation, that GPR50 promotes neurite outgrowth in number and size (Grunewald et al, 2009). Also TGFβ signaling is implicated in axon specification during development (Yi et al, 2010) and plays a role in synaptogenesis in adults (Krieglstein et al, 2011; Poon et al, 2013). Probably, the crosstalk of both might have important function during brain development or in the adult brain in synaptic plasticity, which is often altered in mental disorders as depression (Marsden, 2013).

### 3.2.2. GPR50 and energy homeostasis

Another function of GPR50 is associated with energy homeostasis maintenance. Animals that lack GPR50 have a higher metabolic rate and less weight gain when fed a high fat diet (Ivanova et al, 2008). This is also consistent with the high levels of GPR50 expression in the DMH (Lee et al, 2012), a region important for regulation of the energy status of an organism. A recent study demonstrated an implication of TGFβ signaling, notably Smad3, in the development of obesity and diabetes (Yadav et al, 2011).

### 3.2.3. GPR50 and wound healing

With its effect to promote cellular migration, epithelial-to-mesenchymal transition and the production of ECM components, TGFβ has a positive influence on wound healing processes in the organism (O'Kane & Ferguson, 1997). Interestingly, also GPR50 has been found upregulated in hypertrophic scar (Zhang et al, 2010) during wound healing. Probably, we could expect synergistic effects of GPR50 and the TβRI in tissue repair in the organism. A more detailed analysis of wound healing and migration in the absence and presence of GPR50

in addition to our performed experiments and the establishment of relation to active TGFβ signaling can tell, if this is an important pathway to influence tissue restoration after injury.
Further potential for physiological relevance of the crosstalk of GPR50 with TβRI dependent signaling will hopefully be gained by figuring out whether we can apply our concept to other members of the type I receptor family, whose expression levels are more precise and implicated in the regulation of specific functions in distinct organs.
An identification of *in vivo* relevance of this GPR50-mediated activation mechanism will provide insight where this alternative mode of TβRI activation takes place. In a (far) future, this might be the subject to therapeutic strategies that can directly target this crosstalk via genetic or chemical therapy.

## 4. Perspectives

The identification of a new regulatory mode for the TβRI by GPR50 bears therapeutic potential for the future. Even though GPR50 is an orphan receptor, synthetic ligands that act on GPR50 might be designed. These could probably regulate its activity and affect in turn the activity of their binding partners.
Our findings implicate also the possibility, that ectopic expression of GPR50 can restore TβRI activity in case of ligand or TβRII dysfunction. Strategies, related to gene therapies, either through vector based exogenous expression in target tissue or artificial modulation of gene expression are possible options to induce GPR50 expression when its actions might be advantageous for a cell.
In contrast, we can also think of cases, where a constitutive activity might be undesired, as in late stages of cancer or the fibrotic development of a tissue repair processes. In this case, strategies that target the activity of the complex, like inverse agonists or impede with complex formation like interfering peptides might be useful.

# EPILOGUE

The exploration of regulatory mechanisms of signal transduction pathways has been one of the major interests in research during the last years. Regulatory proteins are often responsible for the establishment of flexibility and specificity and context-dependent signaling of a pathway. The work prepared for this thesis led to the discovery of a new regulator of TGFβ signaling and showed for the first time of a 7TM protein and TβRI on the level of the plasma membrane. The orphan 7TM protein GPR50 can form a complex with the TβRI, which induces its constitutive activation. Surprisingly, this mechanism seems to be different from the commonly established activation mechanism for TGFβ signaling, that implies signal propagation from extracellular ligand binding to TβRII then engaging the TβRI. In this work, we describe the previously unappreciated idea, that the TβRI can be activated in absence of ligand and TβRII. Thus, we not only identified another regulator for the TGFβ signaling pathway that can assists in fine tuning of the TGFβ signaling pathway, but we also describe a new mode of activation for the TβRI. Hence, we add a new facet to the various aspects of TGFβ signaling which will hopefully help to better understand functioning of this signaling pathway in physiological and pathologicacal circumstances.

Moreover, we determine a new role for GPR50 that is comforting its ligand-independent function and strengthens its position as a modulatory protein in the signaling network of a cell. For the first time, we also provided evidence that the two frequent human variants of GPR50 have differential functional effects on a molecular level.

Future research that aims for further detailing of the interaction mechanism and where and when we might require a GPR50-induced ligand-independent TGFβ signaling will surely provide more information on the formation, regulation and physiological relevance of the GPR50/TßRI complex.

Taken together, our work reveals new features of TGFβ signaling, identifies another function for the orphan 7TM protein GPR50 and establishes a functional relevance for both human variants thus adding some new aspects to the understanding of cellular signal transduction concepts that open new ways for therapeutic strategies.

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# ANNEX